A Proof of Concept Randomized Control Trial of Atorvastatin as a Stable Therapy in Bronchiectasis

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This thesis is dedicated to my husband Punit, for all his love, support and believing in me and to my parents Rita and Alok for their encouragement.

CONTENTS

DECLARATION

This thesis describes work undertaken in the University of Edinburgh's Centre for Inflammation Research, Queen's Medical Research Institute and Centre for Infectious Diseases and the Department of Respiratory Medicine, Royal Infirmary of Edinburgh from November 2010 to August 2013. The work described in this thesis has been my own and the writing of this thesis has been entirely my own undertaking. The randomized control trial is currently in press with the Lancet Respiratory Medicine. This work has not previously been submitted for a higher degree or other professional qualification.

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ABSTRACT

Background

Bronchiectasis is characterized by chronic cough, sputum production and recurrent chest infections. The pathogenesis is poorly understood but pulmonary pathology shows excess neutrophilic airways inflammation. Accumulating evidence suggests that statins have pleiotropic effects, including modulation of innate and adaptive immune system and anti-inflammatory effects and therefore is a potential novel anti inflammatory therapy for patients with bronchiectasis.

Aim

The aim of this thesis was to (i) establish if atorvastatin could break the vicious circle of infection and inflammation in bronchiectasis and (ii) establish the anti inflammatory mechanisms of statins contributing to this.

Methods

A RCT was conducted to test the hypothesis that aorvastatin could be a potential anti inflammatory therapy in bronchhiecatsis. In this RCT patients aged 18-79 years were recruited. Patients had clinically significant bronchiectasis, which is patients with cough and sputum production when clinically stable, with two or more chest infections in the preceding year and bronchiectasis confirmed on CT scan of the chest. We excluded: current smokers or ex-smokers of less than 1 year, those with a greater than fifteen pack year history or those with predominant emphysema on CT scan; cystic fibrosis; active allergic bronchopulmonary aspergillosis; active tuberculosis; poorly controlled asthma; pregnancy or breast feeding; known allergy to statins; currently on statins or statin use within 1 year; active malignancy; chronic liver disease; patients on long term oral macrolides; patients chronically colonized with *Pseudomonas aeruginosa*.

Sequence generation was done by block randomization of four, by Tayside pharmaceuticals, NHS Tayside, for 30 patients to receive either atorvastatin 80mg or 30 to receive placebo orally, once daily for 6 months. The placebo (lactose) was not matched to the atorvastatin in appearance. Pharmacy directly dispensed study medications to the patients, hence allocation concealment was maintained at all times

to the study investigators. The primary endpoint of this study was a reduction in cough at 6 months compared to baseline as measured by the Leicester Cough Questionnaire (LCQ) score. It is a 19 item self completed quality of life measure of chronic cough which ranges from 3-21, a lower score indicating a more severe cough. The minimum clinically important difference (MCID) is 1.3 Units. The LCQ score is repeatable over 6 months in stable disease. Analysis done was intention to treat. Secondary outcomes included: forced expired volume in one second, forced vital capacity; incremental shuttle walk test; qualitative and quantitative sputum bacteriology; exacerbation frequency; health related quality of life; sputum neutrophil numbers and apoptosis; sputum myeloperoxidase and free elastase activity; sputum-interleukin (IL)-8; systemic inflammation- white cell count, C-reactive protein (CRP) and erythrocyte sedimentation rate, additional systemic inflammatory markers (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and tumor necrosis factor-α) and safety of therapy.

Findings

(i) RCT

There was evidence of a difference in baseline to 6-month change in LCQ between the treatment groups, with a significant improvement in the statin treated group, with a mean difference 2.2, 95% CI for difference (0.5, 3.9) p=0.01.

When analyzed as proportion of improvement in LCQ, in the statin treated group 40% patients had a 1.3 Units or more improvement in the LCQ compared with 17% in the placebo group; difference in proportion 23% (95% CI for difference 1%, 45%), p=0.04.

There was significantly increased number of apoptotic airway neutrophils [mean difference of 8.9 (11.7); p=0.04] with a trend towards a decreased total number of neutrophils in the sputum; p=0.09; in statin treated group.

Ten (33%) patients had an adverse event in the statin group compared to three (10%) in the placebo, difference in proportion 23% (95% CI for difference 3%, 43%), p=0.02. There were however no serious adverse events.

(ii) *In vitro* studies

Statins enhance apoptosis of neutrophils *in vitro* and this is consequent to reduction in stimuli induced increase in calcium flux.

Interpretation

(i) In this proof of concept study, six months of atorvastatin improved cough in bronchiectasis. Multi-centered studies are now needed to assess whether long-term statin therapy can reduce exacerbations.

(ii) Further studies are needed to establish if statins regulate Ca^{2+} flux by altering the extracellular or intracellular pathways.

THESIS

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CHAPTER 1
INTRODUCTION

1.1. BRONCHIECTASIS

"This affection of the bronchia is always produced by chronic catarrh, or by some other disease attended by long, violent, and often repeated fits of coughing."
R.T.H. Laennec.

1.1.1. DEFINITION AND EPIDEMIOLOGY

Bronchiectasis is a common chronic debilitating respiratory condition. First described by Laennec in 1819, then by Osler in the late 1800s, and then further detailed by Reid in 1950s, bronchiectasis has since undergone significant changes in prevalence, etiology, diagnosis and management (Bilton 2008, Luce 1994). Patients suffer daily cough, excess sputum production and recurrent chest infections because of inflamed and permanently damaged airways (Rougin 2008). Pulmonary pathology shows excess neutrophilic airways inflammation, but despite this over two thirds of patients are chronically infected with potential pathogenic microorganisms.

Reid defined bronchiectasis as permanent dilatation of the airways, a term that has stayed with the disease for more than 60 years. Bronchiectasis can be characterised by the pathologic or radiographic appearances. Cylindrical or tubular bronchiectasis is characterised by dilated airways; varicose bronchiectasis (so named because its appearance is similar to that of varicose veins) is characterized by focal constrictive areas along the dilated airways that result from defects in the bronchial wall; and saccular or cystic bronchiectasis is characterized by progressive dilatation of the airways, which leads to formation of large cysts or saccules and always indicative of the most severe form of bronchiectasis (figure 1) (Reid 1950).

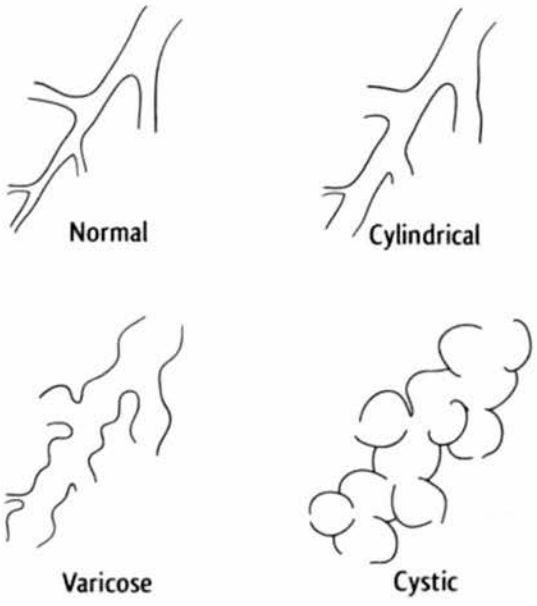


Figure 1. Bronchial dilatation in bronchiectasis as defined by Reid *et al.*

There is no systematic data available on the incidence or prevalence of bronchiectasis. It is generally presumed that with the use of antibiotics in the 20th century there has been a decline in the incidence of bronchiectasis (Barker 2002).javascript:showrefcontent('refrenceslayer'); Data available suggest that the prevalence of bronchiectasis reflects the socioeconomic conditions of the population under study, with significantly lower prevalence in areas where immunizations and antibiotics are readily available. Hence, bronchiectasis remains a major cause of morbidity in countries with limited access to medical care and antibiotic therapy (Singleton et al 2000, Chang et al 2002).

Similarly, in UK there are no recent studies, although chest x-ray features of bronchiectasis in the 1950s suggested a prevalence of 100/ 100 000 (Reid 1950). This is perhaps an under estimation of the actual incidence of bronchiectasis as most of these studies were conducted before the diagnostic methods such as high resolution CT scans were available (BTS guidelines 2010). For instance, the pathological changes of bronchiectasis have been identified using HRCT in up to 15- 30% of patients diagnosed in primary care with chronic bronchitis and chronic obstructive pulmonary disease (COPD) (Patel et al 2004, O'Brien et al 2000). In international studies, bronchiectasis is more common in women and the mean age is in the 60's

although it can affect any age (Reich and Johnson 1992).

1.1.2. PATHOPHYSIOLOGY

The pathogenesis of bronchiectasis is poorly understood. Pulmonary pathology shows excess neutrophilic airways inflammation (Angrill 2001), but despite this over two thirds of patients are chronically infected with potential pathogenic organisms (Angrill 2002). The affected areas may show a variety of changes, including transmural inflammation, oedema, scarring, and ulceration. Patients suffer daily cough, daily sputum production and recurrent chest infections, leading to an inflammatory disease condition. The acute inflammatory response is a protective mechanism that is evolved to eliminate invading organisms and should ideally be self-limiting and lead to complete resolution (Serhan 2007, Houck 1979, Mantovani et al 2011, Medzhitov 2010). However there is failure of resolution of inflammation in bronchiectasis, leading to irreversible damage and dilatation of the bronchial airways with loss of mucociliary function. It is assumed there are intrinsic abnormalities of the innate and adaptive immune systems that predispose to impaired clearance of respiratory pathogens and exaggerated inflammatory responses secondary to abnormal immune regulation. Repeated micro-aspiration of upper airway organisms during recurrent respiratory infections when mucociliary clearance is temporarily compromised may help establish lower airway infection. The driver for persistent neutrophilic airway inflammation in bronchiectasis is unknown, but infection is considered to play a major role (Angrill 2001). This is easy to understand as part of a 'vicious cycle' which was first described by Cole and colleagues and all of our current management strategies are directed towards breaking this cycle (Cole 1984).

1.1.3. VICIOUS CIRCLE

Described by PJ Cole in 1984 (Cole 1984), there is a 'vicious circle' (figure 2) of airways inflammation and bacterial infection in bronchiectasis. The lung is continuously exposed to inhaled pathogen and it is the primary and secondary defense of the lung that maintains sterility of the lung. The excessive neutrophilic airways inflammation leads to damage of the bronchial wall and paradoxically promotes more airways inflammation and bacterial infection creating a vicious cycle (Barker 2002, Cole 1984). During natural resolution, polymorphonuclear neutrophils are required for antimicrobial defense (Medzhitov 2010), but these cells must then apoptose and be removed from the inflammatory site by macrophages (Henneke and Golenbock 2004, Rossi et 2006, Dinarello 2010, Schif-Zuck et al 2011). Is this natural resolution impaired in bronchiectasis? The resolution mechanism is poorly studied and understood in bronchiectasis.

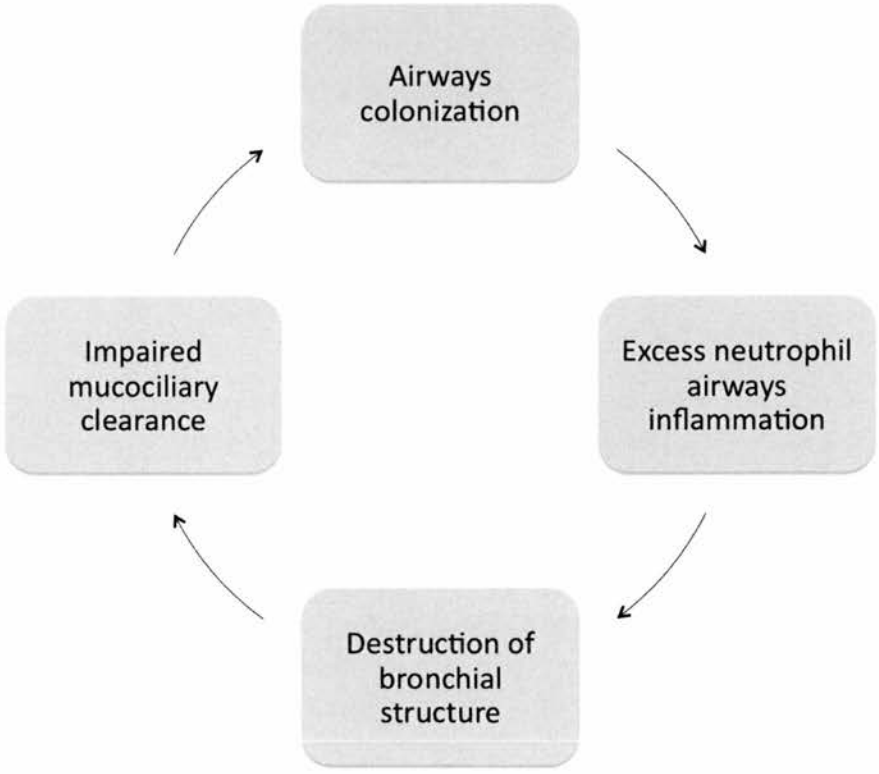


Figure 2. Vicious circle as described by Cole.

1.1.4. AETIOLOGY

Although there are many different disease processes, bronchiectasis is the final pathological end point. All the different etiologies ultimately lead to destruction of the airway epithelium, damage of the mucociliary clearance thereby leading to persistent infection and inflammation. Part of the assessment of patients with bronchiectasis involves identifying where possible the primary insult and allowing specific management. Identifying an underlying cause may limit the need for expensive, invasive or time-consuming investigations and may direct appropriate management. Examples include the commencement of immunoglobulin replacement therapy in patients with common variable immune deficiency and considering oral steroid treatment in those with allergic bronchopulmonary aspergillosis. A significant proportion of adults have bronchiectasis secondary to previous pneumonia or other lower respiratory tract infections but often no cause is identified (BTS guidelines 2010). All other causes are much less frequent. Causes are summarized in table 1.

Etiology	Incidence (%)	History/ Signs	Investigation	Expected findings
Idiopathic	53		Diagnosis of exclusion	
Post infection	29	History of previous infection (e.g. pneumonia; whooping cough; measles; TB);		

Immune defect	8		Immunoglobulin G, A, M and protein electrophoresis. IgG subclasses and antibody response to vaccines (especially *Pneumococcal* and *Haemophilus*).	Decreased values of immunoglobulins, subclasses or functional antibody deficiency.
Allergic bronchopulmonary aspergillosis (ABPA)	7	History of asthma and fleeting infiltrates or proximal bronchiectasis on CXR or CT chest	Full blood count; Total IgE, Ig E to aspergillus, Aspergillus precipitins; 24 hr sputum for aspergillus; HRCT	Raised eosinophils, total IgE and IgE specific to aspergillus. CXR or HRCT evidence of fleeting infiltrates or proximal bronchiectasis.
Aspiration/ Gastro oesophageal reflux (GORD)	4	History consistent with aspiration or reflux	Bronchoscopy if indicated (e.g. foreign body aspiration)	Foreign body/ mucus plugging in bronchus
Rheumatoid Arthritis (RA)	3	History of RA	Autoimmune screen (Anti CCP)	Positive screen
Cystic Fibrosis (CF)	3	Age <40 years,	CF cytogenetics (CFTR gene	CFTR mutations Positive sweat test:

		malabsorption, male infertility; diabetes	mutations); Sweat test	chloride>60mEq/l
Ciliary Dysfunction	1.5	History of situs inversus/ productive cough/ deafness/ infertility	Ciliary motility assessment from one separate site on the respiratory tract	Abnormal ciliary beat pattern
Ulcerative Colitis	<1	History of chronic productive cough, malabsorption; diarrhoea; weight loss; joint pain	Gastro-intestinal investigations (colonoscopy and GI referral if not already done)	Biopsy suggestive of inflammatory bowel disease.
Congenital	<1		N/A	

Table 1. Etiology, clinical findings and relevant investigations in brochiectasis. HRCT= high resolution computed tomography; CFTR= cystic fibrosis transmembrane conductance regulator.

1.1.4.1 Post infectious

Lower respiratory tract infections are associated with the etiology of bronchiectasis and most common is bacterial pneumonia. Pertussis, pulmonary tuberculosis, mycoplasma and viral pneumonia (particularly adenoviruses and measles but also influenza and respiratory syncytial viruses) have, in addition, all been linked directly to permanent lung damage and bronchiectasis (Pasteur et al 2000). Post-tuberculous bronchiectasis is often segmental or lobar in the area primarily affected (Scala et al 2000). In studies investigating the aetiology of bronchiectasis in those over the age of 50, infection was identified as the etiology in more than a third of the cases (Warner 1935).

1.1.4.2. *Mycobacterium tuberculosis* and non-tuberculous mycobacteria

Bronchiectasis may result from pulmonary *Mycobacterium tuberculosis* infection, with the incidence reflecting the prevalence of tuberculosis in the population. It is also increasingly recognised that opportunist mycobacteria are associated with localised or widespread bronchiectasis. Opportunist mycobacteria have been isolated in 2% and 10% of random sputum specimens from patients with bronchiectasis, but the clinical significance is unclear. Patients with Mycobacterium avium complex (MAC) infection may develop bronchiectasis over years. Middle-aged or elderly women are particularly prone to this disease and is known as Lady Windermere disease and this particularly affects the middle lobe or the lingula (Reich and Johnson 1992). However, isolation of an opportunist mycobacterial species should not necessarily be interpreted as pathogenic. Persistent isolation (colonisation) may occur without any change in clinical status. Careful follow-up is mandatory because colonisation can change to infection. Patients should be reassessed if there is a change in clinical features, change in sputum conventional microbial culture, there is a rapid decline in spirometry, or if there are characteristic HRCT changes (exudative 'tree-in-bud' bronchiolitis, mucus plugging, cavitating nodules, rapid progression of broncheictasis; (figure 3)). The species isolated will also influence the likelihood of infection (*M avium complex, M kansasii, M malmoense*), for example mycobacteria such as *M gordonae* maybe just a contaminant. (BTS guidelines 2010).

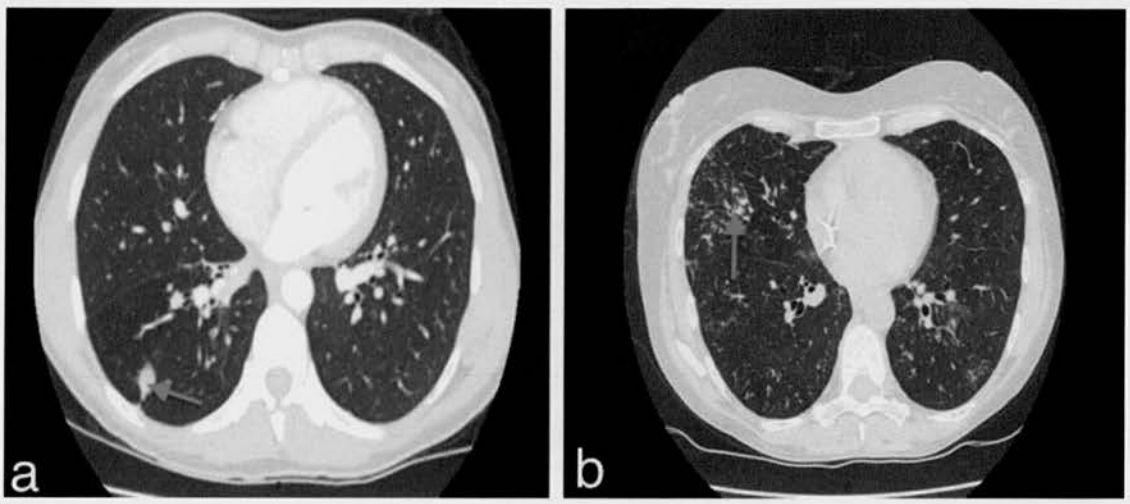


Figure 3. a. HRCT showing tuberculous granuloma in bronchiectasis (as indicated by arrow). 3b. HRCT showing 'tree in bud' appearances in bronchiectasis (as indicated by arrow).

1.2. EXACERBATIONS

Definition of an exacerbation in bronchiectasis

The BTS guidelines recommends antibiotics for exacerbations that present with an acute deterioration (usually over several days) with worsening local symptoms (cough, increased sputum volume or change of viscosity, increased sputum purulence with or without increasing wheeze, breathlessness, haemoptysis) and/or systemic upset.

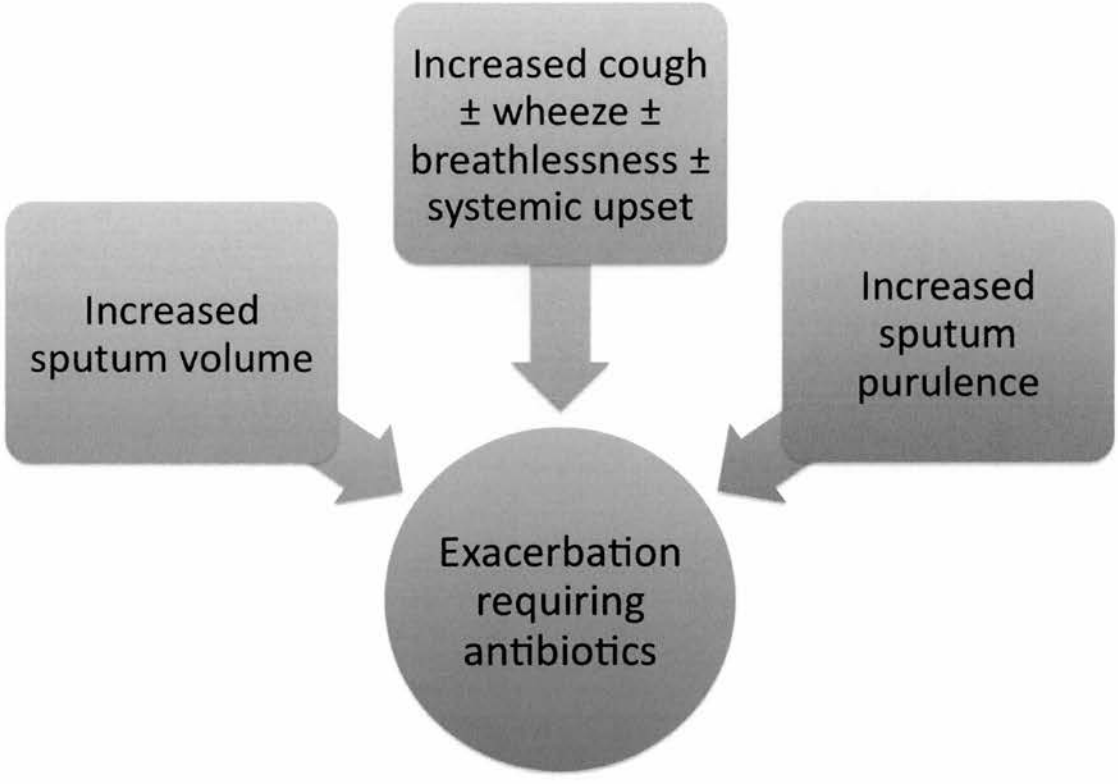


Figure 5. Exacerbations as defined by the BTS guidelines, 2010.

1.3. SPUTUM MICROBIOLOGY AND COLONIZATION

In bronchiectasis, the normally sterile airways are chronically colonised with pathogenic organisms in about 70% of patients. In adults, definitions of colonization have included at least three isolates of an organism over a period of at least 3 months; and at least two isolates 3 months apart over 1 year (BTS guidelines 2010).

Common gram-positive organisms colonising the airways include *Streptococcus pneumoniae* and *Staphylococcus aureus* (both methicillin-sensitive and methicillin-resistant). Gram-negative pathogens include uncapsulated nontypable *Haemophilus influenzae*, *Moraxella catarrhalis*, *Klebsiella*, *Enterobacter* and non mucoid and mucoid *Pseudomonas aeruginosa (*Angrill et al 2002*)*. The type of bacterial infection is important in the determination of the patient's prognosis. Studies have shown that patients colonised with *Pseudomonas aeruginosa* usually have worse symptoms, worse health-related quality of life and accelerated decline in forced expiratory volume over one second (FEV_1) (Martinez-Garcia et al 2007). *Pseudomonas aeruginosa* and other gram-negative organisms are usually found in patients with more severe bronchiectasis. Patients are encouraged to hand in sputum samples for routine microbiology culture as this can guide future antibiotic therapy.

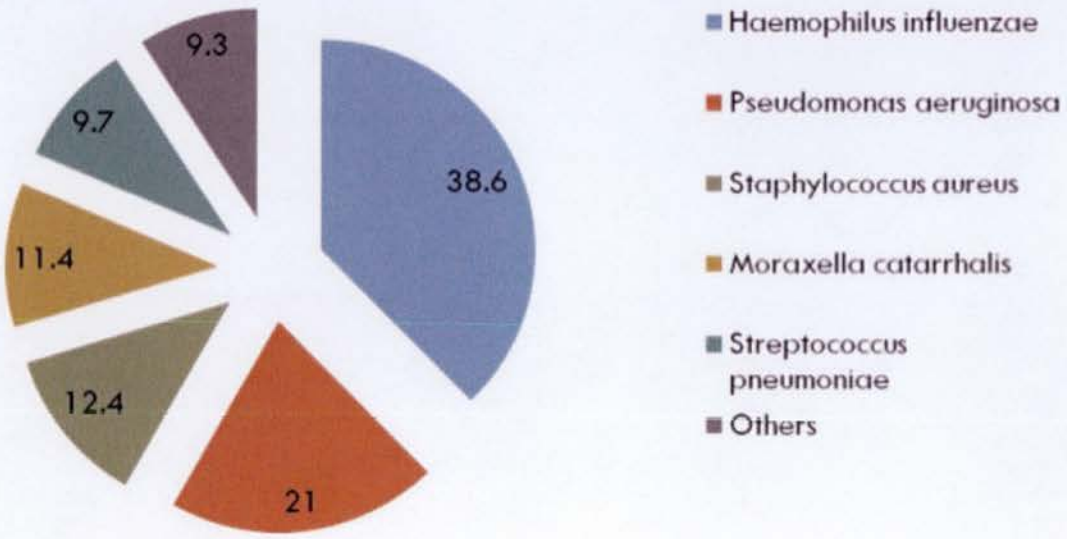


Figure 4. Summary of common pathogens isolated from bronchiectasis patients (Chalmers et al 2012).

1.3.1. *Haemophilus influenzae*

In adults, *H. influenzae* is the most frequently isolated microorganism, with about 35% patients being colonized with it. In bronchiectasis, 39% of the patients are colonized with *Haemophilus influenzae* (Chalmers *et al*). It is a small (1 μm X 0.3 μm), non-motile, pleomorphic, gram-negative coccobacillus. There are both capsulated and non-capsulated (also known as non typeable) strains. It has now been recognized that NTHi (non typeable *Haemophilus influenzae*) is a major cause of respiratory infection, which tends to be chronic and recurrent and includes sinusitis, otitis media, tonsillitis, pneumonia and chronic bronchitis and systemic infection (Murphy 2001). In the context of infection, it has the capacity to live intracellularly especially in macrophages (Craig et al 2002). In chronic bronchitis, studies have shown that large proportions of patients have persistent infection with NTHi and may show extensive invasion of the lung (Moller et al 1998) NTHi is a heterogeneous pathogen with many different strains (Bruant et al 2003) and there is considerable turnover with new strains being acquired periodically (Samuelson et al 1995). In a study done by King *et al*, they confirmed that NTHi was the most commonly isolated *H.influenzae* in bronchiectasis (King et al 2003).

Studies have demonstrated that serum is bactericidal for NTHi. The bactericidal effect of serum on NTHi appears to be mediated through a combination of antibody (IgM and IgG) and complement (King et al 2008, Gnehm et al 1985, Musher et al 1983). This bactericidal action suggests killing by the terminal attack complex of complement with insertion of complement components C5–9 to form a pore in the bacterial membrane. The effect of the membrane–attack complex is well characterized in NTHi, *Neisseria meningitidis* (meningococcal), *Streptococcus pneumoniae* and *Escherichia coli* infections (Figueroa and Densen 1991, Joiner et al 1985).

Some strains of NTHi may be more resistant to serum killing, and Williams *et al* described an invasive isolate of NTHi that was able to inhibit C3 deposition (Williams et al 2001). The role of antibody in respiratory mucosal defence against NTHi is not well defined, although it is likely to be significant. IgA would prevent the binding of NTHi to respiratory mucosa and a major pathogenic mechanism of this bacterium is the production of IgA proteases (Erwin and Smith 2007, Foxwell et al 1998, St. Geme

1997). However, it is known that inflamed bronchi also produce complement and IgG/IgM (Peng et al 2005, Reynolds et al 1997), which may enhance bacterial killing.

There is no widely used effective vaccine for NTHi. There have been problems of antigenic variability and finding a suitable animal model. Most work has focused generally upon enhancing humoral immunity. Developing a vaccine to enhance T lymphocyte responses to NTHi may be important (Williams et al 1998).

Haemophilus influenzae is the most common pathogen isolated from sputum of bronchiectasis patients. Most bronchiectasis patients with chronic NTHi infection are able to mount a highly effective humoral immune response that prevents systemic infection. *H influenzae* die within 3 hours hence to maximize the chances of isolating *H influenzae,* sputum specimens should reach the microbiology laboratory within 3h. This should be done routinely prior to starting antibiotic therapy for exacerbations. The choice of antibiotic and the duration of antibiotic therapy for treating exacerbations with *H.influenzae* infection are summarized in table 2.

1.3.2. *Pseudomonas aeruginosa*

Although *Pseudomonas aeruginosa* has been extensively studied in cystic fibrosis, there is limited data available in the literature about *P.aeruginosa* in non cystic fibrosis bronchiectasis. *P.aeruginosa* is a gram-negative bacillus (non capsulate and non sporing) that affects particularly the lower respiratory tract (Banerjee and Stableforth 2000). Without any treatment, *P. aeruginosa* infection persists in spite of the recruitment of the host's defence mechanisms and leads to decreased respiratory function (Dudley et al 2008). The initial infection is not associated with an immediate and rapid decline in lung function, and early *P. aeruginosa* isolates appear non-resistant to the antibiotic treatment (Geller 2009), giving an opportunity for an early efficient therapeutic intervention (Rosenfield et al 2003). Aggressive and early antibiotic treatments of *P. aeruginosa* infection were shown to increase the life expectancy of CF patients (Geller 2009, van Westreenen and Tiddens 2010). *P. aeruginosa* can form biofilms (Hoiby et al 2010), develop a mucoid phenotype and lack membrane porins (that are essential for antibiotic diffusion) (Nicas and Hancock 1983) or can develop an active drug efflux mechanism (Nikaido 1994).

In bronchiectasis, chronic infection with *P.aeruginosa* is observed in 24-33% (Pasteur et al 2000) patients. In most cases, mucoid *P. aeruginosa* infections cannot be eradicated. However, early aggressive antibiotic treatment is recommended and an attempt should be made to eradicate it (BTS guidelines 2010). Hence it is of utmost importance to send sputum promptly for culture and sensitivity, prior to starting antibiotics. The BTS guidelines recommends that an attempt to eradicate *P.aeruginosa* should be made with high dose oral ciprofloxacin 750mg BD, for 2weeks (BTS guidelines 2010). Failing this, it is recommended to administer intravenous or nebulised anti pseudomonal antibiotic for 2-4weeks (BTS guidelines 2010). However, careful monitoring of renal function and side effects of high doses of antibiotics should be considered and treatment should be based on a balance of benefits outweighing risks. The above treatments recommended are recommended are based on expert opinion only and RCTs are needed to determine whether early interventions influence outcome.

Chronic *P.aeruginosa* infection is associated with poorer lung function, decreased quality of life and frequent hospital admissions (Evans et al 1996, Wilson et al 1997). Martinez and colleagues (Martinez et al 2008) conducted a study in 67 patients with stable bronchiectasis and followed them up for 2years, conducting spirometry, microbiological analysis of sputum and recorded number of exacerbations over the study period. They detected chronic colonization with *P. aeruginosa* was a independent predictor of forced expiratory volume in 1-second (FEV_1) decline [odds ratio (OR), 30.4; 95% CI, 3.8-39.4; p=0.005].

1.3.2.1. *P. aeruginosa* and biofilms

One of the important features of *P.aeruginosa* is its ability to form biofilms. These are defined as a sessile community characterised by cells that are irreversibly attached to a surface or interface, or to each other, and embedded in a matrix of extracellular polymeric substance exhibiting an altered phenotype (Donlan and Costerton 2002). Biofilms protect the bacterial population against host defence and increase the tolerance to antimicrobials. It may be difficult for neutrophils to penetrate into mucus plaques (figure 6). Though being aerobic, *P. aeruginosa* within biofilm is able to survive in the hypoxic environment of the mucus plug, which diminishes its sensitivity to antibiotics (Boucher 2004). This is emphasised by their ability to form in the lungs of chronically ill patients, on catheters and on prosthetic valves, often requiring prolonged periods of antibiotic therapy to eradicate the organism. Links between biofilm formation and quorum sensing have been well established (Popat et al 2008, deKievit 2009). In environments deplete of iron, flat biofilms are formed with wild-type *P aeruginosa*, which had previously grown in the classical mushroom form in an iron replete medium (Patriqin et al 2008). The primary source of carbon as a nutrient also influences the structure of the biofilm, which differs from those in which glucose is the main source rather than citrate. This is thought to be due to differences in the motility of the bacterial population influenced by the primary carbon source (Kausen et al 2003). By manipulating the quorum sensing of *P aeruginosa* populations and thereby creating mutants, the type and shape of biofilm formed is also influenced, with mutants forming flat biofilms compared with wild type (Davies et al 1998).

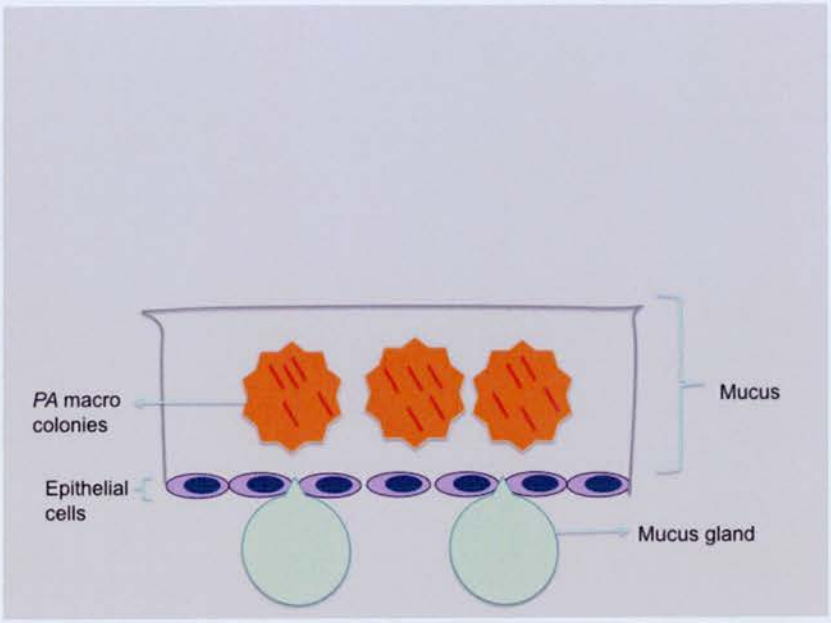


Figure 6a. Flat or iron deplete biofilm.

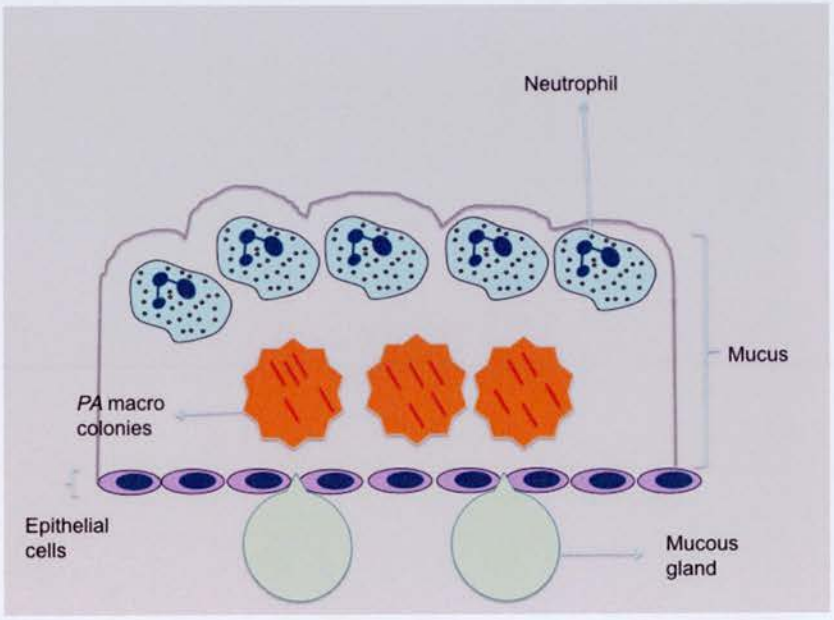


Figure 6b. Mushroom biofilm.

1.3.2.2. Quorum sensing and biofilm development

A number of specific bacterial factors enhance the ability of the pathogen to evade host defences. These include adherence, the ability to impair ciliary clearance and damage the epithelial surface (which enhances both), and avoidance of phagocytosis (Nicod 1999). Therefore, the balance between the innate and adaptive immune response and bacterial factors that avoid this response will result in reduced bacterial clearance and facilitate proliferation leading to colonization.

An area of increasing interest and research in bacterial behavior is quorum sensing. This complex cell-to-cell signaling mechanism of bacterial populations modulates their behavior and ability to survive, colonize and potentially invade the host. Quorum sensing is defined as the capacity to detect extracellular, small-molecule signals and to alter gene expression in response to bacterial population densities that reach a threshold concentration. These signaling pathways modulate various functions of the bacteria, including their metabolism, expression of virulence factors and biofilm formation (Bjarnsholt and Givskov 2007).

Many common bacteria contain quorum-sensing genes. Much of the research to date has specifically examined the quorum-sensing system employed by *P aeruginosa*. It is difficult to eradicate and therefore its behaviors mediated by quorum-sensing systems and potential interactions with the host defence are likely to be of major importance.

The quorum sensing of Gram-negative bacteria is predominantly via the well characterised N-acyl homoserine lactone (AHL) signalling system (Popat et al 2008, Bjarnsholt and Givskov 2007). The AHL-based circuits are encoded by two signalling systems operating with specific signalling molecules, termed the Las and Rhl system. The two systems operate with specific signal molecules: 3-oxo C12-HSL for the lasR-encoded receptor and C4-HSL for the rhlR-encoded receptor (Popat et al 2008, Bjarnsholt and Givskov 2007). These systems also interact with host defences. Telford *et al* (Telford et al 1998) demonstrated that the *Pseudomonas* quorum-sensing signal molecule N-(3 oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) had immunomodulatory activity in addition to its recognised role in regulating virulence. Ritchie *et al* (Ritchie et al 2005) showed direct effects on T cells and a role of this

molecule in the initial stages of T-cell activation and Smith *et al* (Smith et al 2002) demonstrated (in mice) that the quorum-sensing systems of *Pseudomonas* could induce inflammatory mediators *in vivo* via T cells, promoting the production of the inflammatory cytokine γ interferon.

In addition, 3-oxo-c12-HSL induces apoptosis in the neutrophils and macrophages. Tateda *et al* (Tateda et al 2003) proposed a mechanism of chronic *Pseudomonas* infection in the lungs involving quorum sensing whereby the formation of biofilms created a favourable environment for *Pseudomonas* survival. They suggested that 3-oxo-C12-HSL induced the production of neutrophil chemotactic factors (IL-8 in humans and macrophage inflammatory protein-2 in mouse models). This sets in motion a chain of events leading to tissue destruction and accelerated neutrophil apoptosis, all of which contribute to the persistence of *Pseudomonas* in the lungs. In the presence of 3-oxo-C12-HSL neutrophil apoptosis is accelerated, therefore reducing bacterial killing capacity by these cells. Incubating neutrophils with 3-oxo-C12-HSL was shown to increase markers of apoptosis. It was proposed that apoptotic neutrophils also provide nutrients for the bacteria, facilitating their proliferation. This concept is outlined in Fig 7. It is not known whether these *Pseudomonas* products are active in the airways of colonized patients with bronchiectasis and further studies are clearly needed to establish whether this process is important in establishing and maintaining colonization of *P aeruginosa* in bronchiectasis.

The choice of antibiotic and the duration of antibiotic therapy for treating exacerbations with *P.aeruginosa* infection are summarized in table 2.

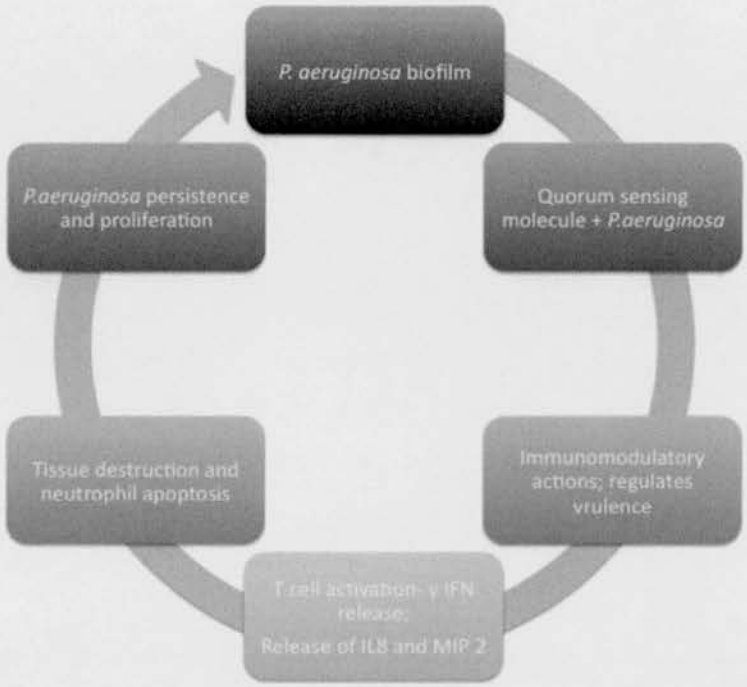


Figure 7. Quorum sensing and biofilm development in *P. aeruginosa*.

While many investigations into bronchiectasis contain microbiological information, only a few offer a comprehensive cross-sectional analysis of bacterial isolation. Difference in methodology and population that is being studied makes it difficult to compare the studies. However, it can be concluded that *P. aeruginosa* colonization is common in bronchiecatic adults and that chronic colonization is associated with more severe bronchiectasis and worse disease prognosis.

1.3.3. Other potential pathogenic microorganisms (PPM)

The other PPMs of significance in bronchiectasis are *S.pneumoniae*, *M.catarrhalis*, enteric gram-negative organisms like *E.Coli* and *Klebsiella* and *S.aureus* (both methicillin sensitive and methicillin resistant).

1.3.3.1. *Streptococcus pneumoniae*

In bronchiectasis, *S. pneumoniae* is detected in about 1-22% of patients (Angrill et al 2002). It is grown on blood agar and to maximise the chances of isolating *S. pneumoniae*, specimens should reach the microbiology laboratory within 3 hours. Exacerbations are treated with oral antibiotics (Amoxicillin 500mg TDS or Clarithromycin 500mg BD) for 14 days (BTS guidelines 2010). The BTS guidelines recommend long term antibiotic therapy for *S. pneumoniae* colonisation with Amoxicillin 500mg BD as first line and Clarithromycin 250mg BD as second line of treatment (BTS guidelines 2010). Combination antibiotics are not recommended for treating exacerbations with *S.pneumoniae* (BTS guidelines 2010).

S. pneumoniae are lancet-shaped, gram-positive, facultative anaerobic bacteria with over 90 known serotypes, of which 23 are included in the pneumococcal vaccine Pneumovax ®. Most *S. pneumoniae* serotypes have been shown to cause disease, but only a minority of serotypes produce the majority of pneumococcal infections. Each serotype is characterized by an individual polysaccharide capsule, which functions as a key virulence factor (Hyams et al 2010). Antibody to cell wall constituents of the pneumococcus becomes attached to the surface of the organism, and in turn binds complement. The presence of the capsule prevents iC3b and the Fc of immunoglobulins on the bacterial cell surface from interacting with their receptors on the surface of phagocytic cells, with the result that the organisms remain extracellular (Musher 1992). The capsule is also crucial for colonization, prevents mechanical removal by mucus (Nelson et al 2007), and can also restrict autolysis and reduce exposure to antibiotics (Van der Poll et al 2009).

Another important pneumococcal virulence factor is the exotoxin pneumolysin (PLY). PLY of most pneumococcal strains is cytotoxic to mammalian cells by forming large pores into membranes (Mitchell and Mitchell 2010). Pneumococci

are common inhabitants of the respiratory tract and may be isolated from the nasopharynx of 5-70% of adults, depending on the population and setting. *S.pneumoniae* is the most common cause of community acquired pneumonia and bacterial meningitis. In bronchiectasis, *S.pneumoniae* is frequently isolated in sputum cultures, varying between 1-22%.

S. pneumoniae remains a common pathogen and a major cause of morbidity and mortality. *S. pneumoniae* infections are estimated to cause 500,000 cases of pneumonia, 55,000 cases of bacteremia, and 6,000 cases of meningitis annually in the United States (Williams et al 1998). The clinical course of *S. pneumoniae* infections is affected by a number of factors including the site and severity of infection, the underlying health of the patient, and the adequacy of antimicrobial therapy. Therefore, it is not surprising that estimates of mortality are reported to range from <1% to >50%.

Initially, all *S. pneumoniae* isolates were exquisitely susceptible to penicillin (MIC, ≤0.06 mg/mL), and this antibiotic served as the drug of choice. Beginning in the 1960s, however, clinical resistance to penicillin and other agents began to be reported. The clinical significance of penicillin resistance in *S. pneumoniae* was not appreciated until 1967, when Hansman and Bullen (Hansman and Bullen 1997) recovered a penicillin-resistant *S. pneumoniae* isolate (MIC, 0.6 mg/mL) from a patient with hypogammaglobulinemia and bronchiectasis.

The choice of antibiotic and the duration of antibiotic therapy for treating exacerbations with *S.pneumoniae* infection are summarized in table 2.

1.3.3.2. *Moraxella catarrhalis*

It is a gram-negative, aerobic, oxidase-positive diplococcus. The endotoxin of *M catarrhalis,* a lipopolysaccharide may play a role in the disease process. Some strains of *M catarrhalis* have pili or fimbriae, which may facilitate adherence to the respiratory epithelium. Some strains produce a protein that confers resistance to complement by interfering with the formation of the membrane attack complex. An important feature of *M. catarrhalis* is that at least 90% of isolates produce a β-lactamase with activity against β-lactams such as penicillin and the amino penicillins (Fung et al 1994).

Studies have demonstrated that *M. catarrhalis* is isolated in 1-20% of patients with bronchiectasis. Its persistence within the respiratory tract is most likely aided by its capacity to invade respiratory epithelial cells, inactivate complement and form biofilms (Murphy et al 2009). In addition, more than 90% of *M. catarrhalis* isolates are β-lactamase producers and resistant to ampicillin.

Other resident bacterial flora, including α-haemolytic streptococci, can inhibit the growth of *M. catarrhalis*, *H. influenzae* and *S. Pneumoniae*. This competitive balance is altered by antibiotics and also during an acute viral respiratory infection where numbers for all three pathogens are increased (Murphy et al 2009). *M. catarrhalis* is acquired early in life and is a prominent coloniser of the upper respiratory tract in infants and children, before decreasing substantially during the adult years.

Antibiotic recommended for acute exacerbations is summarized in Table 2 and long term antibiotic treatment for *M. catarrhalis* is summarized in Table 2.

1.3.3.3. *Staphylococcus aureus*

It is a facultative anaerobic gram-positive coccus. Staphylococci are nonmotile, non–spore-forming, and catalase-positive bacteria. The cell wall contains peptidoglycan and teichoic acid. Most strains of *S. aureus* are sensitive to the more commonly used antibiotics. Those that are sensitive to methicillin are termed methicillin-sensitive *Staphylococcus aureus* (MSSA). Methicillin-resistant *Staphylococcus aureus* (MRSA) and MSSA only differ in their degree of antibiotic resistance. Over the last 20 years, community and hospital infections with *S. aureus* have risen-in particular with MRSA and more recently vancomycin resistant *S. aureus*.

1.3.3.4. Other enteric gram-negative organisms (*Klebsiella, Enterobacteriaceae*)

Angrill and colleagues they showed that bronchial colonisation by PPMs are common in patients with bronchiectasis in a stable clinical situation and in more than 60% of these patients the distal airways were colonised. They also concluded that sputum culture is an adequate tool for the evaluation of bronchial colonisation in bronchiectasis and that early diagnosis of bronchiectasis (before the age of 14), evidence of varicose-cystic bronchiectasis and a FEV_1 of <80% predicted are risk factors for the presence of PPMs in the airways (Angrill et al 2001). Additionally, in a further study done by the same group, they performed bronchoalveolar lavage (BAL) in 49 patients with bronchietasis and 9 controls and found that in patients with clinically stable bronchiectasis: (*1*) there is an active neutrophilic inflammatory response in the airways that is present in patients with sterile bronchi but is exaggerated in patients with PPMs colonizing the airways; (*2*) colonizing bacteria act as an inflammatory stimulus with greater bacterial load producing greater inflammatory response; (*3*) the bronchial inflammatory response was compartmentalized and could not be accurately evaluated in blood samples (Angrill et al 2002). Recently, Chamlers *et al*, investigated this further in interventional and observational studies in bronchiectasis and concluded that high airway bacterial loads were associated with airway and systemic inflammation and a greater risk of exacerbations. They concluded that short and long-term antibiotic therapies reduce markers of airways and systemic inflammation (Chalmers et al 2013).

Organism	Recommended first line treatment	Recommended second line treatment	Length of treatment
Streptococcus pneumoniae	Amoxicillin 500mg tds (po)	Clarithromycin 500mg bd (po)*	7-14 days
***Haemophilus influenzae*, β-lactamase negative**	Amoxicillin 500mg tds (po)	Doxycycline 100mg bd (po) Or Clarithromycin 500mg bd* (po) Or Ciprofloxacin 500 mg* bd (po) Or Ceftriaxone 2G od* (IV) or 1g bd if administering as iv bolus.	7-14 days
***Haemophilus influenzae*, β-lactamase positive**	Co-amoxiclav 625mg TDS (po)	Doxycycline 100mg bd (po) Or Clarithromycin 500mg bd* (po) Or Ciprofloxacin 500mg bd* (po) Or Ceftriaxone 2G od* (IV) or 1g bd if administering as iv bolus.	7-14 days
Moraxella catarrhalis	Co-amoxiclav 625mg TDS (po)	Doxycycline 100mg bd (po) Or Clarithromycin 500mg bd* (po)	7-14 days

		Or Ciprofloxacin 500mg bd* (po) Or Ceftriaxone 2G od* (IV) or 1g bd if administering as iv bolus.	
***Staphylococcus aureus* (MSSA)**	Flucloxacillin 500mg QDS (po)	Clarithromycin 500mg BD (po)* Doxycycline 100mg BD or Coamoxiclav 625mg TDS	7-14 days
***Staphylococcus aureus* (MRSA) Oral preparations**	Rifampicin 300mg bd (po) + Trimethoprim 200mg bd (po)	Rifampicin 300mg bd (po)+ Doxycycline 200mg od (po) Third line Linezolid 600mg bd (po)	14 days 14 days
***Staphylococcus aureus* (MRSA) Intravenous preparations**	Vancomycin* or Teicoplanin* 400mg od (iv)	Linezolid 600mg bd (po)	14 days
Coliforms e.g. Klebsiella, Enterobacter	Oral Ciprofloxacin 500mg bd* (po)	Intravenous Ceftriaxone 2G od or 1g bd if administering as iv bolus.	7-14 days
Pseudomonas aeruginosa	Oral Ciprofloxacin 500mg bd* (po) (750mg bd in more severe	Monotherapy Intravenous Ceftazidime 2G tds* Or Tazobactam/Piperacillin 4.5G tds*	14 days

	infections)	Or Meropenem 2G tds* Or Aztreonam 2G tds* Combination therapy: The above can be combined with gentamicin* (refer to BNF) or colistin 1-2 MU tds* (under 60 kg, 50 000-75 000 units/kg daily separated into 3 divided doses) OR oral ciprofloxacin 500mg BD*.	

Table 2. Choice of antibiotic and duration of treatment.

*dose needs adjusted in renal impairment

1.4. DO LONG-TERM ORAL ANTIBIOTICS INFLUENCE OUTCOME?

The BTS guidelines 2010 recommend long term antibiotics in:

1. Patients having ≥ 3 exacerbations per year requiring antibiotic therapy or patients with fewer exacerbations causing significant morbidity should be considered for long-term antibiotics.
2. In the first instance, high doses should not be used, to minimise side effects.
3. The antibiotic regimen should be determined by sputum microbiology when clinically stable
4. Long-term quinolones should not be used until further studies are available.
5. Macrolides may have disease-modifying activity and preliminary data suggest the need for a large randomised controlled trial. There have been subsequently 3 RCTs that have been conducted with macrolides in bronchiectasis and all three have shown that long term macrolide treatment leads to decrease in exacerbation frequency (Wong et al 2012, Serisier et al 2013, Altenburg et al 2013). These trials are discussed in details in section 1.4.1.

Organism	**Recommended first line treatment**	**Recommended second line treatment**
Streptococcus pneumoniae	Amoxicillin 250 mg bd	Clarithromycin 250 mg bd
Haemophilus influenzae, ß-lactamase negative	Amoxicillin 250 mg bd	Clarithromycin 250 mg bd
Haemophilus influenzae, ß-lactamase positive	Co-amoxiclav 375 mg tds	Clarithromycin 250 mg bd
Moraxella catarrhalis	Co-amoxiclav 375 mg tds	Clarithromycin 250 mg bd
Staphylococcus aureus (MSSA)	Flucloxacillin 500 mg bd	Clarithromycin 250 mg bd

Table. 3. Long term antibiotic treatment as recommended by the BTS.

1.5. DIAGNOSIS

1.5.1. RADIOLOGY

1.5.1.1. Chest X ray

The chest X-ray is usually normal unless patients have severe bronchiectasis. However, a normal chest x ray does not rule out bronchiectasis. Digital acquisition devices are capable of producing x-rays with improved visualisation of, for example, bronchiectatic airways behind the heart, with the added potential of radiation dose reduction (Young et al 1991). However, unless disease is severe, the radiographic signs of bronchiectasis are usually inconspicuous. Characteristic chest X-ray appearances include crowding of bronchi, parallel line opacities (tram lines) caused by thickened dilated bronchi, ring opacities or cystic spaces as large as 2 cm in diameter resulting from cystic bronchiectasis, sometimes with air-fluid levels, and oligaemia as a result of a reduction in pulmonary artery perfusion (figure 8). In terms of specificity, the chest x-ray of a patient with COPD showing bronchial wall thickening (tramline and ring shadows) and large volume lungs may be erroneously interpreted as indicating bronchiectasis. In summary, although a baseline chest x ray is recommended in all patients with bronchiectasis, they are not routinely indicated for follow up of patients with no change in symptoms (BTS guidelines 2010). Additionally, there is very little correlation during an exacerbation of bronchiectasis and chest x ray changes.

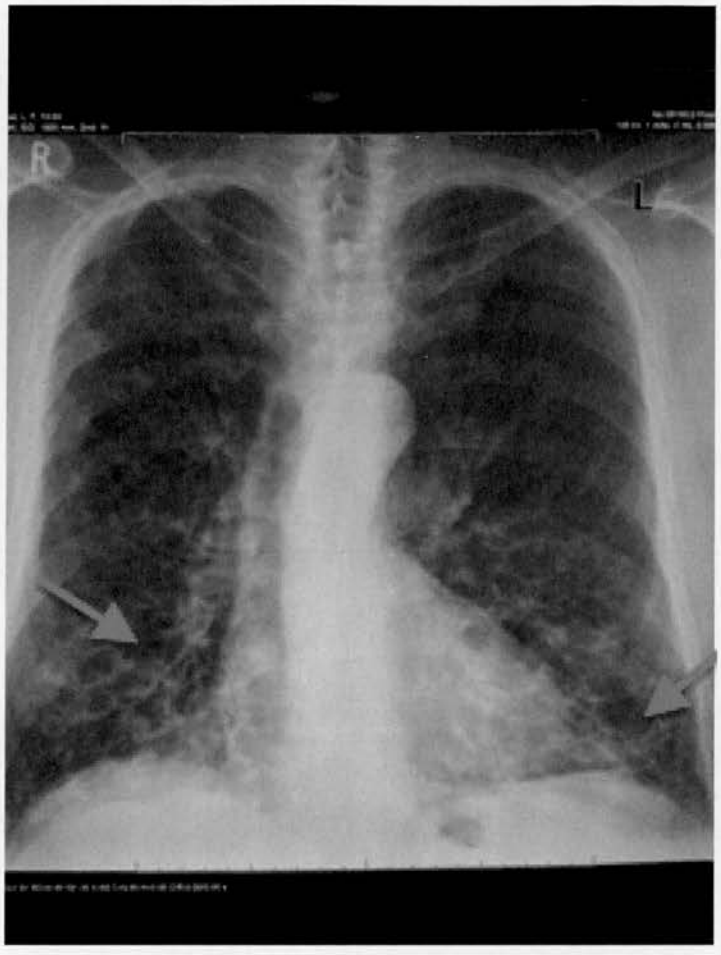


Figure 8. Cystic bronchiectasis- as indicated by arrows.

1.5.2.High Resolution CT Scan

The current gold standard for diagnosing bronchiectasis is HRCT of the chest. The appropriate high resolution CT study is a non contrast study with the use of 1.0-1.5mm window every 1cm with acquisition times of one second, reconstructed with the use of a high spatial frequency algorithm during full inspiration (Munro et el 1990). Bronchiectasis is diagnosed when there is bronchial wall dilatation, defined as the internal lumen of the bronchus being greater than that of the accompanying pulmonary artery, described as the 'signet' ring sign (figure 9). Characteristic features include, lack of tapering toward the periphery producing cylindrical bronchiectasis (figure 10), varicose constrictions along the airway (figure 11) and ballooned cysts at the end of a bronchus (figure 12). Cylindrical bronchiectasis is by far the most common bronchiectasis identified on CT. The usefulness of categorising bronchiectasis into cylindrical, varicose or cystic subtypes is limited, but cystic bronchiectasis usually denotes longstanding and more severe disease.

Bronchial wall thickening is a usual but inconsistent feature of bronchiectasis. Problems with this variable feature have been widely debated and the definition of what constitutes abnormal bronchial wall thickening remains unresolved (BTS guidelines 2010).

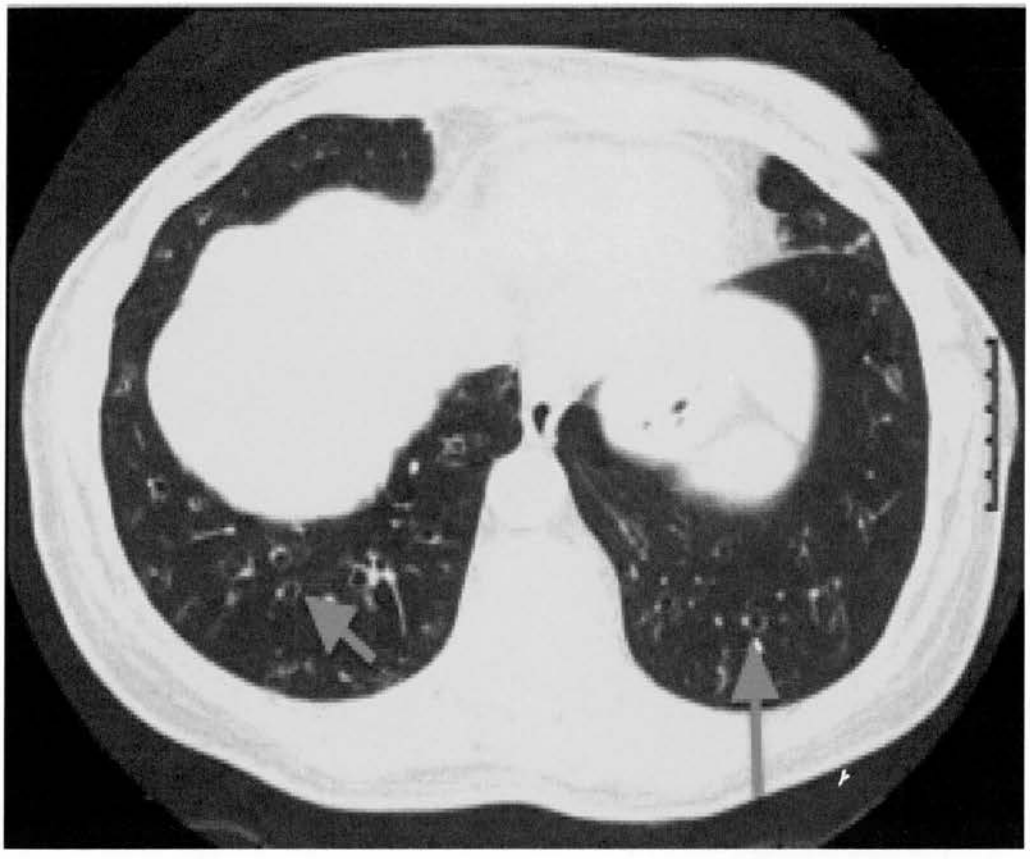


Figure 9. Signet ring (as indicated by arrows).

Non specific findings include consolidation or infiltration of a lobe with dilatation of the airways, thickening of bronchial walls, mucus plugs and enlarged lymph nodes (Thomas and Blaquiere 1993). Mucus secretions within bronchiectatic airways are generally easily recognisable as such. The larger plugged bronchi are visible as lobulated or branching opacities. Such airways are usually seen in the presence of non-fluid filled obviously bronchiectatic airways. Mucus plugging of the smaller peripheral and centrilobular airways produces V- and Y-shaped opacities, the so-called 'tree-in-bud' pattern (Gruden and Webb 1995, Collins et al 1998, Franquet and Fern 1999).

In patients with ABPA there is fleeting infiltrates and ultimately patients can develop proximal bronchiectasis. The characteristic distribution with *M. avium* complex infection often involves the middle lobe or lingula (Cartier et al 1999) and typically shows bronchiectasis, nodes and 'tree in bud' appearences. With the use of high-resolution CT, dilated airways may be seen in other diseases, adding to the confusion

regarding the diagnosis of bronchiectasis. Airway dilatation is associated with asthma, chronic bronchitis, and pulmonary fibrosis (so-called traction bronchiectasis). This thesis however deals with clinically significant bronchiecatsis, which is radiological diagnosis and clinical features in keeping with bronchiectasis.

In summary, HRCT is the radiological investigation of choice in bronchiectasis, with bronchial wall dilation being most characteristic of bronchiectasis.

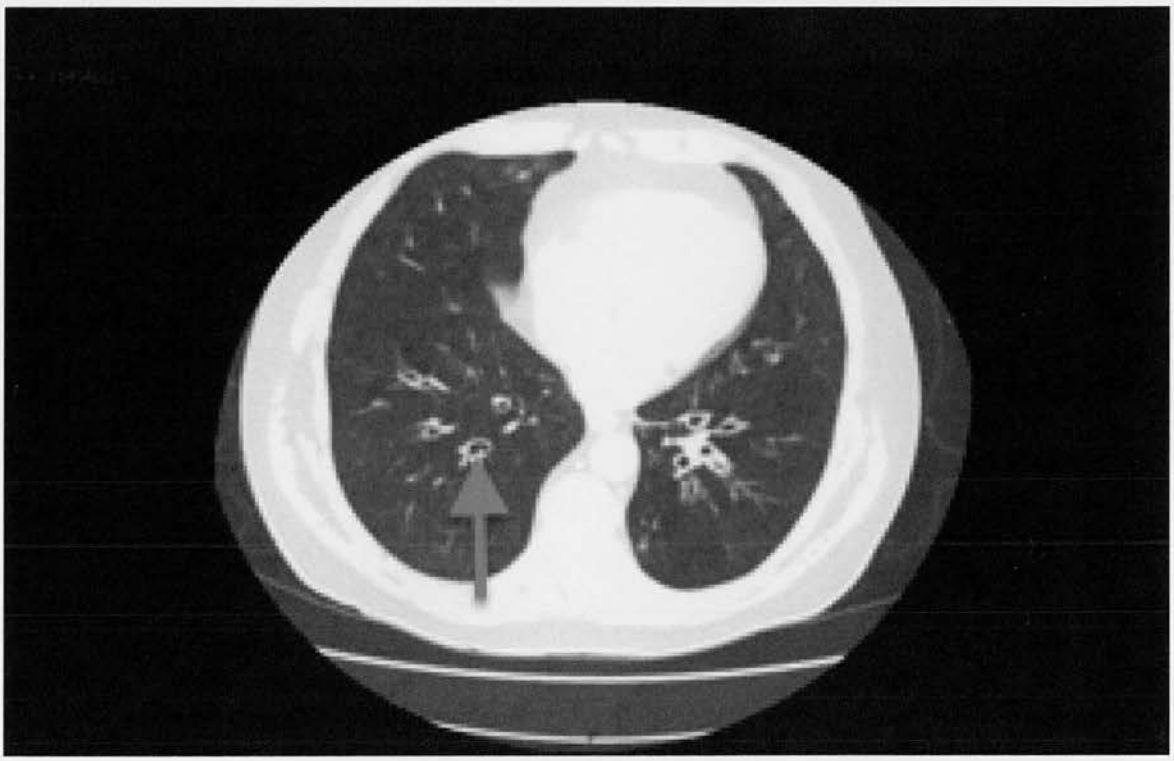


Figure 10. Tubular bronchiectasis (as indicated by arrows).

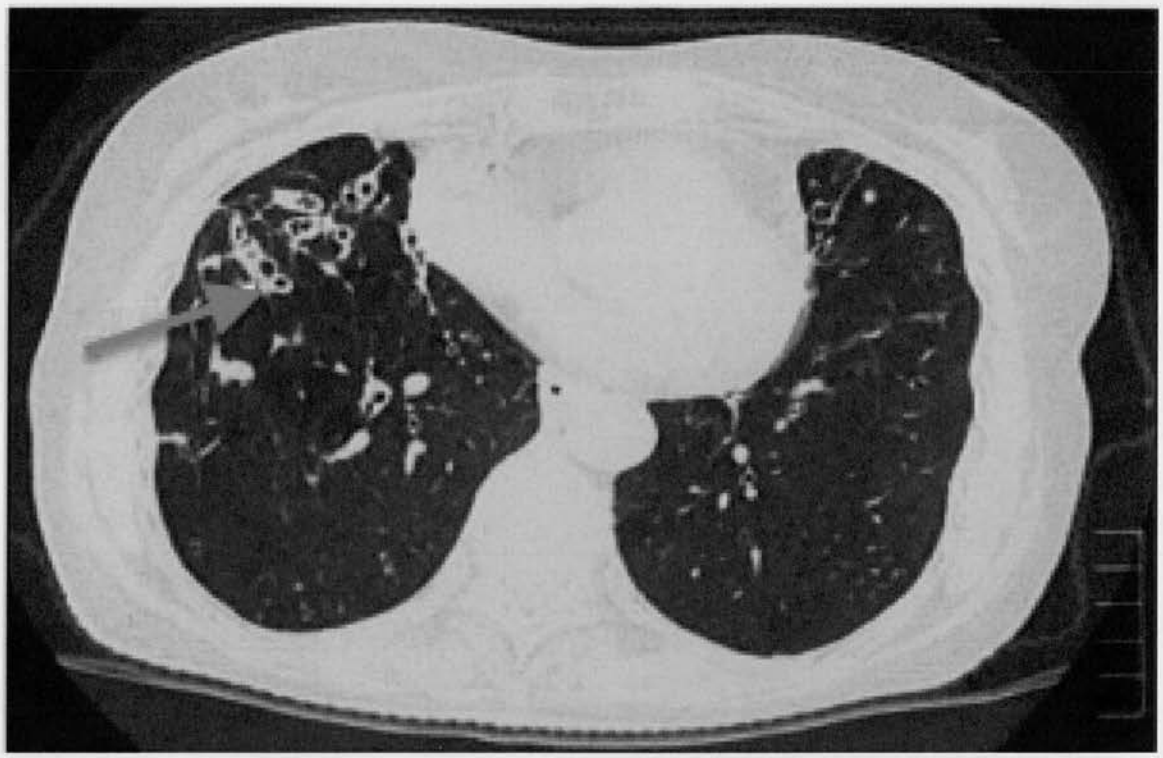


Figure 11. Varicose bronchiectasis (as indicated by arrows).

Figure 12. Cystic bronchiectasis (as indicated by arrows).

1.5.2 CLINICAL FEATURES OF BRONCHIECTASIS

1.5.2.1. Cough and sputum

Cough is the commonest symptom of bronchiectasis and occurs in >90% patients (BTS guidelines 2010). The cough is productive of sputum daily in 75-100%, intermittent in 12-20% and non-productive in 5-8% (Stockley et al 2001). In bronchiectasis, cough occurs due to overproduction of secretions and impaired mucociliary clearance of secretions. Mucus hypersecretory states in bronchiectasis results usually from either hypersecretion of stored mucin or hypertrophy and hyperplasia of goblet cells.

24 hour sputum volume measurement is perhaps a more accurate assessment of volume produced, but is entirely dependant on patient compliance. Sputum purulence is dependant on the release of myeloperoxidase and is classified as mucoid (clear or grey), mucopurulent (pale yellow) and purulent (dark yellow or green) (Stockley et al 2001). Sputum purulence is related to radiological appearances on CT scan and purulent sputum is associated with cystic bronchiectasis on HRCT (O'Brien et al 2000) and correlates well with the presence of potential pathogenic microorganisms (Murray et al 2009).

1.5.2.2. Dyspnoea, hemoptysis, pain and fever

Dyspnoea is present in about 70% of patients and correlates inversely to FEV_1. Haemoptysis can occur with exacerbations but major haemoptysis an infrequently occur and usually arises from an aberrant blood vessel from the bronchial arteries and can sometimes require interventions such as bronchial embolisation or lobar resection. Patients can present with pleuritic chest pain and fever with exacerbations.

1.5.2.3. Infective exacerbations

As previously mentioned, the BTS guidelines recommended antibiotics for exacerbations that present with an acute deterioration (usually over several days) with worsening local symptoms (cough, increased sputum volume or change of viscosity, increased sputum purulence with or without increasing wheeze, breathlessness, haemoptysis) and/or systemic upset (BTS guidelines 2010). Number of exacerbations can vary but from the national BTS audit the number of exacerbations in a secondary

care cohort is 2 per year (Hill et al 2012). Studies have shown that recurrent exacerbations lead to poor quality of life (Wilson et al 1998).

1.5.2.4.Clinical signs on bronchiectasis

The most characteristic physical finding of bronchiectasis is coarse crackles on auscultation of the lung bases, and is found in about 70% of patients (BTS guidelines 2010). Some patients may also have airflow obstruction and or clubbing.

1.5.2.5. Impact on quality of life

Assessment of psychological symptoms and quality of life has shown that patients with bronchiectasis have increased anxiety and depression scores, increased fatigue and lower quality of life. The St George's Respiratory Questionnaire has been validated for use in bronchiectasis (Wilson et al 1997). Levels of depression are related to dyspnoea score, and patients colonised with *Pseudomonas* have lower quality of life than those colonised with other microorganisms. Symptoms, particularly cough, may also impact on family members. The Leicester Cough questionnaire has been validated for use in bronchiectasis and a lower score indicates worse cough severity and poorer quality of life (Murray et a 2009).

Diagnosis is based on a combination of clinical, radiological and sputum microbiology results. Patients are put in severity categories of mild, moderate or severe bronchiectasis based on a combination of signs and symptoms as outlined in the table 4 below. This serves more as a guide to stratify patients into severity rather than as an assessment tool.

Severity	Sputum purulence	Sputum microbiology	HRCT features
Mild	No sputum or mucoid sputum	Not chronically colonised	Mild tubular bronchiectasis ≤3 lobes
Moderate	Mucoid or mucopurulent	Chronically colonised with organisms other than *Ps. aeruginosa*/enteric gram negative organisms or MRSA	Tubular bronchiectas ≥3 lobes or varicose bronchiectasis
Severe	Purulent	Colonised with *Pseudomonas aeruginosa* or other enteric gram negative organisms or MRSA.	Cystic bronchiectasis

Table 4. Guide to assessing severity of bronchiectasis.

1.6. IMMUNE SYSTEM OF THE LUNG AND BRONCHIECTASIS

The innate immune system is the immediate, non-specific and first line of defense against invading organisms. Key cells of the innate immune response include neutrophils, macrophages, eosinophils, basophils, dendritic cells and γδ T cells. The mucociliary escalator is lined by ciliated and mucus-producing cells. Particles >2–3 μm impact on the mucus covering epithelial cells in the upper airways and bronchi and are removed by a combination of normal mucociliary clearance and cough (Nicod 1999, Loebinger et al 2009). Airway secretions also contain several anti-infective proteins and polypeptides, including the bacteriostatic proteins lysozyme and lactoferrin. Secretory leucoprotease inhibitor (SLPI), produced by the mucous glands, macrophages and epithelial cells, has antifungal, antiviral and antibacterial properties (Rogan et al 2009). Lactoferrrin, SLPI, lysozyme and defensins are (among others), derived from recruited neutrophils in addition to submucosal glands (for lactoferrin) (Rogan et al 2009). Secretary IgA is the main antibody isotype in mucosal secretions in the respiratory tract that contributes to the initial defence mechanisms, while IgG predominates in the plasma (Hill et al 2000).

If these primary clearance mechanisms are overwhelmed by a high bacterial load, a secondary defense system is activated. Animal experiments clearly demonstrate this relationship between bacterial load and activation of the secondary host response in that a low bacterial load leads to bacterial clearance, but when the bacterial load is greater than 10^6 colony-forming units/ml (cfu/ml) there is a neutrophilic host inflammatory response (Hiemstra 2007). Our recent paper in bronchiectasis shows that this response occurs at $\geq 10^5$ cfu/ml (Chalmers et al 2012).

Failure of the local host defenses results in microbial colonization, establishing the 'vicious cycle' described by Cole and colleagues. Airway epithelium responds by releasing antimicrobial peptides and inflammatory cytokines into the airways. In human airway epithelial cells the main classes of antimicrobial peptides produced are the β-defensins and the cathelicidins (LL-37 in humans). Chemokines and cytokines such as interleukin (IL)-8 are released into the submucosa (Bals and Hiemstra 2004) and initiate the inflammatory reaction, leading to recruitment of phagocytes (neutrophils, monocytes and macrophages), dendritic cells and lymphocytes (which

contribute to the adaptive response) (Weng et al 2010). The airways become inflamed leading to leakage of serum proteins including circulating antibodies and complement, therefore increasing local antibacterial potential.

1.7. KEY INFLAMMATORY CELLS IN BRONCHIECTASIS

1.7.1. NEUTROPHILS

Excessive neutrophilic airways inflammation is the central feature of bronchiectasis. This paradoxically both promotes bacterial colonization and perpetuates damage to the airways creating a vicious cycle of bacterial colonization and inflammation (Cole 1984). The lung is a unique vascular and externally exposed organ that is subject both to PMN-mediated inflammation and PMN-opposed infection.

Granule proteins from activated neutrophils, such as azurocidin and α-defensins, alter directly permeability changes (Serhan 2007, Houck 1979). Moreover, proteases of neutrophilic origin such as neutrophil elastase have been regarded to be important in degradation of surfactant proteins, epithelial cell apoptosis, and coagulation. In addition, neutrophils produce vast quantities of reactive oxygen (ROS) and nitrogen (RNS) species like O_2^- and NO through their oxidant-generating systems such as the phagocyte NADPH oxidase and nitric oxide synthase (NOS), respectively. Besides their important antimicrobial effector function, neutrophil-derived oxidants promote deleterious pro-inflammatory effects thus being a major cause of neutrophil-dependent tissue injury (Morel et al 1991).

1.7.1.1. Key functions of neutrophils relevant to bronchiectasis

1.7.1.1.1. Recruitment and migration

Recruitment is driven by chemoattractants, with IL8, leukotriene (LT) B_4, interleukin-1 beta, C5a and tumour necrosis factor (TNF) alpha believed to be of significance (Mikami et al 1998). Elevated levels of these pro-inflammatory cytokines have been demonstrated in bronchiectasis airway secretions (Stockley and Bayley 2000). Neutrophil detection of chemotactic stimuli leads to a co-ordinated process of cell signalling, cytoskeletal rearrangement and changes in surface receptor expression to facilitate migration. Key to the process of transendothelial migration is the expression of the integrins CD11/CD18 on neutrophils and the expression of adhesion molecules on endothelial cells, principally intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and the selectins (Downey et al 2009, Cowburn et al 2008).

Neutrophil migration occurs predominately at the border of endothelial cells where modifications of cell junctions allow this. The cell adhesion molecules, platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31) and junctional adhesion molecule (Martin-Padura et al 1998, Muller et al 1993, Vaprociyan et al 1993) are involved in neutrophil transmigration. Migration occurs via PECAM-1/PECAM-1 interaction while maintaining the permeability barrier of the endothelial cell monolayer (Muller et al 1993) The endothelial surface density of ICAM-1 is important in regulating this migration (Yang et al 2005).

Neutrophil adhesion to pulmonary endothelial cells and migration into the lung may occur by CD11/CD18 dependent or CD11/CD18 independent mechanisms (Doerschuk et al 2000). Different stimuli within the lung can determine whether CD18 is required for neutrophil migration into the lung. It is felt that stimuli from gram negative bacteria require CD18, as 75–80% of neutrophil migration is inhibited by CD18 antibodies (Doerschuk et al 2000, Ramamoorthy et al 1997).

Once neutrophils migrate to the site of infection, neutrophils perform three key actions: phagocytosis of the bacteria, release of products of pre formed granules and release of reactive oxygen species-all three actions are directed towards killing of bacteria.

1.7.1.1.2. Neutrophil phagocytosis

The internalization of bacteria by neutrophil and formation of a phagosome is facilitated by opsonization. Opsonization is a process where the Fab portion of the IgG antibody binds to the bacteria and the Fc (receptors are FcγRIIa and FcγRIIIB) portion then binds to the phagocyte by specific receptors. Fcγ receptor ligation leads to extension of the pseudopod around the bacteria, which finally engulfs it completely. Both intracellular and extracellular enviroment is important to regulate neutrophil function (Whitters and Stockley 2012). What is known in bronchiectasis, is that peripheral blood neutrophils function normally in bronchiectasis (Watt et al 2004). Our group demonstrated (unpublished data) that the airway neutrophils do not function normally in bronchiectasis- however we do not know the mechanism for this but hypothesize that the complex airway environment with the overwhelming

bacterial load, inflammatory cytokines and chemokines could all in part contribute to the impaired neutrophil function. This needs to be explored further.

1.7.1.1.3. Release of products of degranulation

Granule proteins formed during neutrophil development and released upon activation is one of the key functions of neutrophils. Changes in the cytosolic calcium are required for granule secretion (Lew et al 1996) and for fusion of the granule to the neutrophilic phagosome (Jaconi et al 1990). The granules contain enzymes, host defense proteins with receptors, signaling proteins and adhesion molecules that are expressed on the cell surface upon activation. Azurophilic granules or primary granules release MPO, defensins, neutrophil elastase, proteinase 3 and cathepsin G, among others. Secondary granules release lactoferrin, cathelicidin and tertiary granules release gelatinase acetyltransferase and lysozyme. Excessive degranulation and release of MPO and neutrophil elastase leads to host tissue damage (Mitchell et al 2008).

1.7.1.1.4. Release of superoxide anion

During phagocytosis, neutrophils increase the oxygen consumption by using up NADPH oxidase and releasing superoxide anion ($O2^-$). The $O2^-$ then dismutates to form H_2O_2, releasing MPO from primary neutrophilic granules (Morel et al 1991). This oxidative burst is critical for bacterial killing but simultaneously MPO causes oxidative damage to the epithelial cells mainly through formation of the cytotoxic oxidant HOCl (Cantin et al 1987, Cantin et al 1993, Worlitzesh et al 2009). Elevated MPO levels have been found to correlate to disease severity in bronchiecatsis (Hill et al 2000). The respiratory burst results in the release of ROS to facilitate bacterial killing. The release of uncontrolled ROS by neutrophils may however also lead to damage to surrounding tissues, amplifying the lung disease process (Tung et al 2009). It had previously been thought that ROS exert a direct toxic effect on ingested pathogens. However Reeves *et al* (Reeves et al 2002) suggested that the respiratory burst sets in motion events, which result in creating a favorable environment for proteases such as elastase to digest the contents within the phagocytic vacuole (figure 13). By optimising conditions via ion flux and pH change, the enzymes released from the cytoplasmic granules become more active, facilitating the destruction of ingested pathogens.

As NADPH oxidase is key for respiratory burst, patients with chronic granulomatous disease (defect in one of the subunits of NADPH oxidase genes) are unable to produce ROS and thereby lead to life threatening bacterial infections (Goody et al 2008). In 2006, King *et al* demonstrated that in bronchiectasis, neutrophils have normal phagocytic capacity but impaired oxidative burst as compared to controls (King et al 2006). However, in contrast, Pasteur *et al* failed to detect any difference in oxidative burst between bronchiectatic patients and controls (Pasteur et al 2000). It remains therefore an important abnormality that needs to be explored further in bronchiectasis.

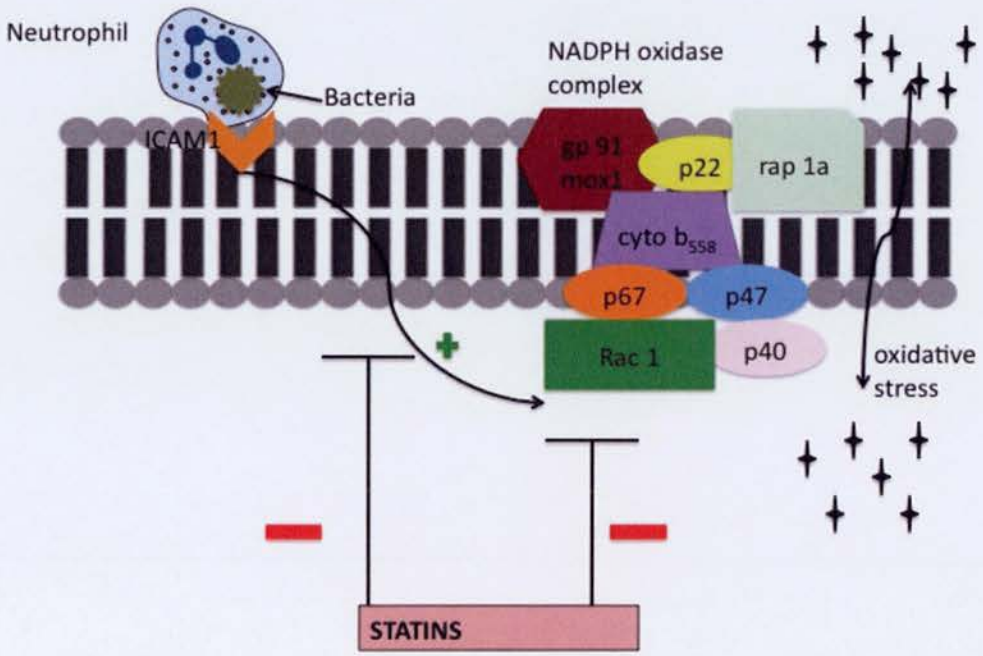


Figure 13. Schematic representation of NADPH oxidase enzyme, reactive oxygen species generation and the proposed role of statins. The integral membrane of the phagocyte consists of 2 subunits: p22 and gp91 which respectively produce the smaller and larger chain of the cytochrome-b_{558}. Two cytosolic subunits: p67 and p47; a p40 accessory protein and a Rac-GTP binding protein then translocate to the cell membrane upon activation to form the NADPH oxidase complex which generates a respiratory burst. Superoxide can react to form hydrogen peroxide and hypochlorous acid, which together participate in bacterial killing *(Adapted from Assari 2006).*

1.7.1.1.5. Apoptosis

It has been well established that there is a key role for apoptosis, or programmed cell death, in the regulation of inflammation and the host immune response. The process of apoptosis, which is distinct from necrosis (toxic cell death), involves a series of coordinated morphologic changes in the affected cell, causing its demise and subsequent recognition and removal by scavenger phagocytes (Wylie et al 1980). Characteristically, apoptosis initially induces cytoplasmic shrinkage associated with membrane blebbing (zeiosis), followed by chromatin condensation and DNA fragmentation. Although cells usually manifest these changes sequentially when undergoing apoptosis, it is now clear that these events may occur independently under the control of separate and distinct metabolic pathways (Duffin et al 2010).

Among the leukocytes, mature human neutrophils have the shortest life span and die rapidly via apoptosis *in vivo* and *in vitro*, resulting in the demise of the entire population within 72h (5-10). As neutrophils proceed through apoptosis, functional activity declines. Apoptotic neutrophils lose CD16 (FcyRIII) expression (I l, 12) and demonstrate a reduced ability to degranulate, generate a respiratory burst, or undergo shape changes in response to external stimuli such as the chemotactic bacterial peptide formyl-methyl-leucyl-phenalalanine or fMLF (Sasmono 2007). Although neutrophils appear to be committed to death via apoptosis, it is now clear that the life span and functional activity of mature neutrophils can be extended significantly by proinflammatory cytokines, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, TNF-α and IL-2 (Burdon 2008).

Although elevation of cytosolic-free Ca^{2+} has been associated with apoptosis in a variety of cell types (Whyte et al 1993), transient elevations of cytosolic free Ca^{2+} induced by low doses of calcium ionophores have been reported to inhibit apoptosis in neutrophils *in vitro* (Whyte et al 1993). Other experiments employing inhibitors of tyrosine kinases and phosphatases suggest a possible role for tyrosine phosphorylation events in the signal transduction pathway mediating neutrophil apoptosis (Erttmann et al 2014). Furthermore, it has been shown that intracellular acidification precedes, and

may be causally related to, the development of morphologic features characteristic of apoptosis in neutrophils maintained in short term culture (Erttmann et al 2014).

It is also known that the Fas (APO-I; CD95)/ Fas-ligand (FasL) system represents an important cellular pathway responsible for the induction of apoptosis in diverse tissues. Fas is a widely expressed 45-kDa type I membrane protein member of the TNF/nerve growth factor family, which mediates apoptosis following interaction with agonistic anti-Fas IgM Ab or FasL (Wajant et al 2002). FasL is a 40-kDa type II protein member of the TNF family, which includes TNF-α, TNF-β (lymphotoxin), CD40 ligand, and CD30 ligand (Wajant et al 2002). In contrast to Fas, the expression of FasL is relatively restricted in its tissue distribution (Wajant et al 2002) but can be induced in mature T cells following stimulation with para-Methoxyamphetamine or PMA, ionomycin, anti-CD3, or bacterial antigen (Wajant et al 2005). Furthermore, it has been shown that activated T cells can secrete a functionally active soluble form of FasL that is capable of inducing apoptosis in other cells susceptible to Fas-induced apoptosis (Wajant et al 2005). The relative contributions of the proapoptotic and pro inflammatory functions of Fas/FasL sytem to the pathogenesis of lung disease and the effects of inhibiting this pathway, remains poorly understood.

Binding of Fas to its natural ligand FasL, can trigger apoptosis via activation of caspase 8. FasL exists in a membrane bound form and a soluble form, both of which can activate Fas. In addition to triggering apoptotic pathways, the activation of Fas can also lead to NF-κB translocation and cytokine production (Kreuz et al 2004).

FasL expression is detected on the surface of mature human neutrophils, but not on monocytes or eosinophils suggesting that neutrophils are susceptible to Fas induced cell death.

There is limited data available from two studies in the literature on apoptosis of airway neutrophils in bronchiectasis. Vandivier *et al* (Vandivier et al 2002) concluded that sputum of bronchiectatic patients have more apoptotic neutrophils in comparison to sputum obtained from patients with chronic bronchitis. However, Watt *et al* demonstrated that there were low levels of apoptotic neutrophils in induced sputum as

compared to the findings by Vandivier and colleagues (Watt et al 2004). They found no significant differences in the levels of apoptotic neutrophils at the beginning and end of an exacerbation treated with antibiotics. There was however a reduction in the total number of sputum neutrophils and serum levels of IL-8, TNF-α, neutrophil elastase (NE) and CRP. In summary, few studies have investigated neutrophil apoptosis in bronchiecatsis and of the two key studies described to date, the opinion remains divided.

1.7.2. MACROPHAGES IN BRONCHIECTASIS

Little is known about the role of macrophages in bronchiectasis. One of the key functions of macrophages is efferocytosis or "burying'' the dead (apoptotic) neutrophils (Serhan 2012). It is well recognized that activated neutrophils must firstly 'switch off' by undergoing apoptosis to limit the inflammatory process and then be cleared by macrophages to prevent secondary necrosis and release of granular products, and thereby promote resolution of inflammation (Koedel et al 2009).

In bronchiectatic patients, Vandivier *et al* demonstrated that neutrophil elastase cleaves phosphatidylserine on the surface of apoptotic cells, preventing efferocytosis by macrophages (Vandivier et al 2002). Further studies are needed to explore this in bronchiectasis.

1.7.3. EPITHELIAL CELLS

1.7.3.1. Airway host defense and damage

The airway mucociliary clearance system is a key component of primary host defence in the lungs, responsible for maintaining airway sterility and health. The system comprises of a mucus gel layer that lines the airway lumen trapping pathogens, inhaled toxins and cellular debris as it is swept by beating cilia from the distal airways to the proximal airways for expectoration and clearance.

In health, the respiratory tract is lined by equal numbers of ciliated columnar epithelial cells and secretory cells. The epithelial cells each have 200 cilia, approximately 7μm length that rest in a thin layer of periciliary fluid. The cilia beat in a 2 stage process, an effective beat where their tips engage with the airway mucus sweeping it through the airways and a recovery beat in the opposite direction where their tips disengage and recover (Purkinje 1835, Lucas 1934). Alveoli produce surfactant to maintain bronchial patency. Bronchioles are lined with ciliated epithelium and secretory cells that produce a thin mucus layer for the airway lumen. The airway mucus is composed predominantly of water (97%) and mucins (3%) and accumulates as it is transported through the larger airways, trachea and pharynx for expectoration.

Dilated, damaged bronchial wall function impairs clearance of mucus and excessive mucus accumulation causes the airways to be persistently exposed to toxic insults and pathogens. In bronchiectasis, the airways are permanently damaged and the normal mucociliary mechanism is believed to be impaired.

1.7.3.2. Abnormal ciliary function

In order to ensure effective mucus transport, the cilia must beat at a normal rate, in a consistent direction and in a coordinated fashion (Eliezer et al 1970). Studies have used ciliated nasal epithelium as a non-invasive means of assessing ciliary function in the respiratory tract. Ciliary beat frequency is significantly slower in bronchiectasis (Rutland and Cole 1981). This finding has been confirmed in a more recent study of 152 patients with idiopathic bronchiectasis which found ciliary beat frequency to be

significantly slower than healthy controls (Tsang et al 2005b). The purulent sputum in bronchiectasis contains excessive quantities of elastase due to the persistent neutrophilic airways inflammation. Such purulent sputum from patients with bronchiectasis has been shown to slow ciliary beat frequency on normal ciliated nasal epithelium in healthy controls. The addition to the sputum of an elastase inhibitor (α1-antitrypsin) resulted in no significant change in ciliary beat frequency over the same time period, suggesting that elastase activity is an independent factor affecting ciliary function (Smallman et al 1984). The effect of elastase on ciliary function and ciliated epithelium has been shown *in vitro* to increase with increasing concentrations of elastase in the sputum with progressive disruption of the epithelium and slowing of ciliary beat frequency at higher levels (Amitani et al 1991). The effect of other inflammatory molecules on ciliary function in bronchiectasis is unknown and needs further study. The effect of bacterial products on ciliary function has been investigated *in vitro*. Exposure of ciliated nasal epithelium to the supernatants of *Pseudomonas aeruginosa* and *Haemophilus influenzae* over a 4 hour period was associated with significant slowing of ciliary beat frequency and a disorganised beating pattern. A dose related effect was also observed and it may be that the release of a factor or factors by these organisms complements the effect of human neutrophil elastase on ciliary inhibition (Wilson et al 1985).

1.8. MANAGEMENT

In mild bronchiectasis, the mainstay of treatment is regular chest clearance with physiotherapy, annual influenza and prompt treatment of exacerbations. In more advanced bronchiectasis, in addition, there should be consideration of long term antibiotic therapy and or anti inflammatory therapy.

1.8.1. Current controversies in the management of bronchiectasis

To achieve the main treatment goals in bronchiectasis, it is important to break the 'vicious circle' of infection and inflammation. Long term antibiotics and chest physiotherapy are the mainstay of long-term treatment in severe bronchiectasis. However, there are certain caveats that we have to bear in mind. We are faced with the burden of *Clostridium difficile* and Methicillin resistant *Staphylococcus aureus* (MRSA) in addition to antibiotic resistance, with the prolonged use of antibiotics. Consensus on several therapeutic strategies that have been evaluated in cystic fibrosis (CF) and COPD is lacking in bronchiectasis, and a number of controversies need further elucidation. Over the last few years, there has been an international drive to reduce antibiotic usage. There is an urgent need for novel non-antibiotic treatments.

1.9. ROLE OF ANTI-INFLAMMATORY AGENTS IN BRONCHIECTASIS

Bronchiectasis is characterized by permanently damaged airways, excess neutrophilic airways inflammation and despite this, there is persistent bacterial colonization. The kinetics of the establishment of infection and their relationship with the subsequent inflammatory response is poorly understood (Chimel et al 2002). Severity of inflammatory response depends on the interplay between pro-inflammatory cytokines, which are upregulated, and anti-inflammatory cytokines and various cytokine inhibitors, which are released to limit its extent and duration (Wouters 2005). The drivers for persistent neutrophilic airway inflammation in bronchiectasis is unknown. We hypothesize that bacteria are a major driver but there maybe other factors as well, which needs to be explored.

1.9.1 Macrolides in bronchiectasis

In bronchiectasis, several studies have been conducted with macrolides, but only three RCTs (randomized control trials) have been conducted to date, assessing the role of macrolide as an anti-inflammatory agent with immunomodulatory properties in bronchiectasis. Key studies are summarized below.

In an open label study Davies *et al* (Davies et al 2004) investigated the effects of regular azithromycin in patients with recurrent exacerbations, starting at 500mg once daily for 6 days reducing to 250mg once daily for 6 days before regularly taking 250mg three times a week. After 4 months of treatment they recorded a reduction in the number of exacerbations requiring antibiotics and an improvement in symptoms and carbon monoxide gas transfer. Similarly, Cymbala *et al* in their open label study also noted a reduction in exacerbation frequency with azithromycin 500mg twice weekly (Cymbala et al 2005).

Wong (Wong et al 2012) confirmed these findings in the Effectiveness of Macrolides in patients with Bronchiectasis using Azithromycin to control Exacerbations (EMBRACE) trial. This was a randomised double-blind placebo controlled trial of 141 patients randomised to either taking azithromycin 500mg three times a week or placebo for 6 months. They showed a significant reduction in the rate of event-based

exacerbation from 1.57 in the placebo group to 0.59 with the azithromycin group. There was no statistically significant difference in lung function or quality of life.

More recently the Bronchiectasis and long term Azithromycin Treatment (BAT) study in 83 bronchiectasis patients with 3 or more exacerbations in the past year were randomised to either 250mg azithromycin once daily or placebo for 1 year (Altenburg et al 2013). Results showed a significant decrease in exacerbation frequency in the azithromycin group with a longer time to first exacerbation during treatment. FEV_1 and FVC improved with azithromycin as did quality of life as assessed by SGRQ. Sputum microbiology was similar at baseline and at 1 year but significant macrolide resistance was recorded in 88% compared with 26% in the placebo group. *Haemophilus influenzae, Staphylococcus aureus* and *Moraxella catarrhalis* were resistant to macrolides at the end of treatment. Adverse reactions were reported, mainly gastrointestinal symptoms, but not severe enough to discontinue treatment.

The Bronchiectasis and Low dose Erythromycin Study (BLESS) investigated the effect on exacerbations rates and resistance rates post 1-year therapy with low dose erythromycin (Serisier et al 2013). Patients were randomised to low dose erythromycin or placebo for 48 weeks. Results showed a significant reduction in exacerbation frequency, reduced FEV_1 decline but an increase in macrolide resistance. Erythromycin was well tolerated without any evidence of significant adverse effects. However, there was a significant rise in the proportion of erythromycin resistant oropharygeal *Streptococci.*

The key results of the three studies are summarized in table 5.

Parameters recorded	EMBRACE trial (N= 141)	BAT trial (N=83)	BLESS trial (N=117)
Exacerbation frequency	Reduced	Reduced	Reduced
Macrolide resistance at the end of treatment	Not recorded routinely, but 4% developed resistance in azithromycin group.	88% (35% at baseline) in azithromycin group compared to 26% (28% at baseline) in placebo.	Mean change increased to 27% in the erythromycin group compared to 0.04% in placebo.
Gastrointestinal side effects	13% in placebo compared to 27% in azithromycin group.	21% in placebo compared to 54% in azithromycin group.	26% in placebo compared to 29% in erythrromycin group.
QTc	Not recorded.	Not recorded	No change in either group.

Table 5. Comparison of key findings in the macrolide trials.

1.9.2 Limitations of long term macrolide use in bronchiectasis

It is indeed very encouraging to see the results from the above three trials using macrolides as an anti inflammatory agent in bronchiectasis in reducing exacerbations. However, the inherent limitation with the use of long-term use of antibiotics (including macrolides), in respiratory diseases is the emergence of new pathogens and increased antimicrobial resistance against the airway microbiota, which limits the enthusiasm for widespread use of antibiotics such as macrolides in bronchiectasis. About 2-10% of patients with bronchiectasis develop non-tuberculous mycobacteria (NTM) disease. The 1st line of treatment for NTM is macrolides and hence there is a concern of macrolide resistance in this group of patients. It seems pragmatic that macrolide maintenance therapy should be considered in patients that have been carefully selected, experience at least 3 exacerbations annually and have had mycobacterial disease excluded.

1.10. STATINS AS AN ANTI INFLAMMATORY

1.10.1. Mechanism of action: Inhibition of HMG CoA reductase

Statins target hepatocytes and inhibit HMG-CoA reductase, the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor. Statins do more than just compete with the normal substrate in the enzymes active site. They alter the conformation of the enzyme when they bind to its active site. This prevents HMG-CoA reductase from attaining a functional structure. The change in conformation at the active site makes these drugs very effective and specific. Binding of statins to HMG- CoA reductase is reversible, and their affinity for the enzyme is in the nanomolar range, as compared to the natural substrate, which has micromolar affinity (Corsini et al 1999). The inhibition of HMG-CoA reductase determines the reduction of intracellular cholesterol, inducing the activation of a protease that slices the sterol regulatory element binding proteins (SREBPs) from the endoplasmic reticulum. SREBPs are translocated at the level of the nucleus, where they increase the gene expression for LDL receptor. The reduction of cholesterol in hepatocytes leads to the increase of hepatic LDL receptors, that determines the reduction of circulating LDL and of its precursors (intermediate density-IDL and very low density-VLDL lipoproteins) (Sehayak et al 1994). All statins reduce LDL cholesterol non-linearly, dose-dependent, and after administration of a single daily dose (Blum 1994). Efficacy on triglyceride reduction parallels LDL cholesterol reduction (Stein et al 1998).

By inhibiting mevalonic acid synthesis, statins also prevent the synthesis of other important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (figure 14) (Sharpe and Brown 2013). These isoprenoids are necessary for the posttranslational modification and subsequent trafficking of intracellular signaling molecules. In addition, they serve as important lipid attachments for the posttranslational modification of a variety of proteins, including the γ subunit of heterotrimeric G-proteins; Heme-a; nuclear lamins; and small guanosine triphosphate (GTP)-binding protein Ras; and Ras-like proteins, such as Rho, Rab, Rac, Ral, or Rap (Buhaescu 2007). Both Ras and Rho are small GTP-binding proteins, which cycle between the inactive GDP-bound state and active GTP-bound state. In endothelial cells, Ras translocation from the cytoplasm to the plasma membrane is dependent on

farnesylation, whereas Rho translocation is dependent on geranylgeranylation (Patel et al 2003). Statins inhibit both Ras and Rho isoprenylation, leading to the accumulation of inactive Ras and Rho in the cytoplasm. Because Rho is major target of geranylgeranylation, inhibition of Rho and its downstream target, Rho-kinase, is a likely mechanism mediating some of the pleiotropic effects of statins on the vascular wall (Buhaescu 2007). Of significnce is that RhoA-dependent cytoskeleton is a negative regulator of eNOS mRNA stability, and statins up regulate eNOS expression by inhibition of RhoA geranylgeranylation (Buhaescu 2007). Hence, it is now possible to explain some important downstream effects of HMG CoA reduction at a cellular level and how they modify cellular phenotype through non-lipid-dependent mechanisms. In addition, many of these effects can be experimentally reproduced in much shorter time course than would be expected by effects mediated by lipid lowering alone. However, the question remains, do these effects occur in humans - and are they quantitatively relevant?

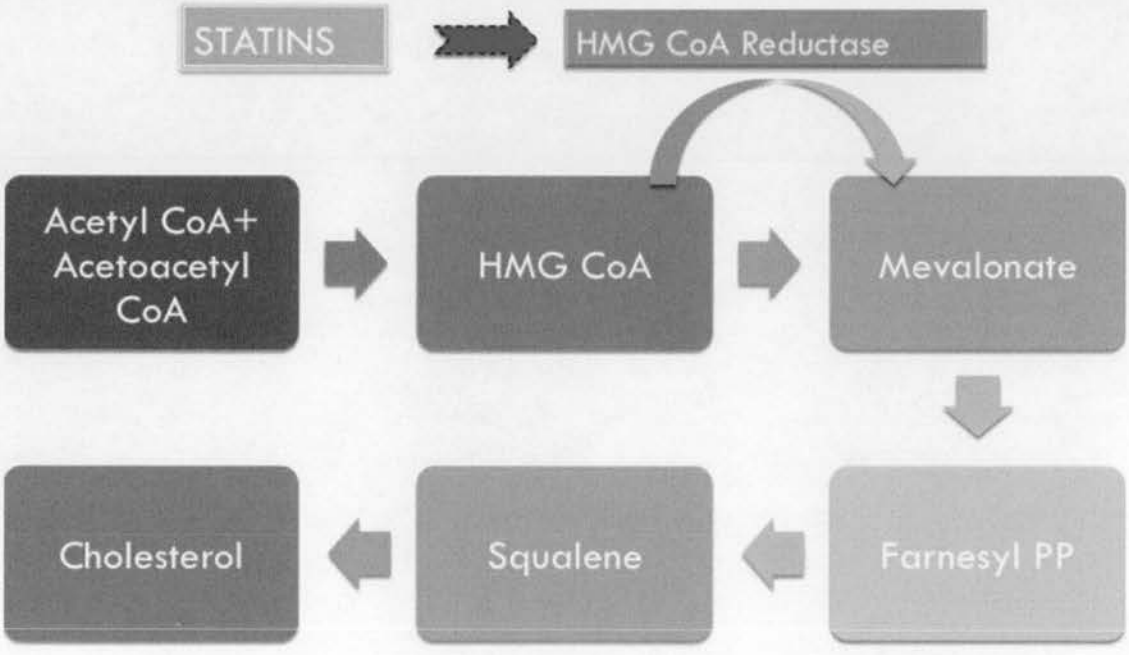


Figure 14. The mevalonic acid pathway.

1.11. STATINS AND THE INFLAMMATORY PROCESS

1.11.1. Statins and sterile inflammation

Statins can reduce the expression and function of molecules on the leukocytes surface (Weber et al 1997). Atorvastatin reduces the number of intimal macrophages, monocyte-chemoattractant protein-1 (MCP-1) and the activation of nuclear factor NFκB in hypercholesterolemic rabbits (Bustos et al 1998). Cytokines receptors are coupled to GTP-bound proteins, and the binding of leukocytes to the endothelium is regulated by G protein. Statins can effect small GTP-ases or trimeric G proteins, by preventing their prenylation and thus reducing the inflammatory response. Statins diminish leukocytes recruitment in postcapillary venules, stimulated by a lipid mediator (platelets activation factor-PAF or leukotriene B_4) in hypercholesterolemic rats (Kimura et al 1997). In addition, statins are capable to inhibit transendothelial migration and chemotaxis of neutrophils, which can explain the anti inflammatory effect of these compounds. Another anti-inflammatory effect of statins on monocytes and macrophages was the decrease of the expression of intercellular adhesion molecule -1 and the secretion of interleukine-6 (IL-6), induced by lipopolysacharides (LPS) (Bellosta 2000).

Statins are being explored as adjunctive therapy for acute lung injury. They have immunomodulatory, antioxidative and anticoagulant properties both *in vivo* and *in vitro*. These effects may be contributed to reduce the markers of systemic inflammation (TNF-α, IL-8, IL-6, MMPs, CRP), decrease mortality, and improve outcomes of in patients with sepsis Viasus et al 2010).

High sensitivity C-reactive protein (hs-CRP) is a non-specific marker of inflammatory disease, which has been shown to be associated with vascular risk independent of cholesterol levels. Treatment with statins lowers hs-CRP levels, and, interestingly, this effect does not correlate with the lowering of LDL-C (Thomas and Mann 1998, Ridker et al 2003, Ridker et al 2001). For example, retrospective analysis of the CARE (Cholesterol and Recurrent Events) trial suggests that elevated CRP predicted risk independent of the lipid profile and patients with high CRP benefited the most from pravastatin treatment, supporting an anti-inflammatory and cholesterol-

independent effect of statins in humans (Ridker et al 2001) (table 6). However, the relation between CRP and atherogenesis needs to be further investigated. In addition to these findings, statins have been shown to reduce graft vessel disease after heart and renal transplantations and to inhibit pro-inflammatory cytokine release (Ridker et al 1999, Southworth and Mauro 1997, Goldberg and Roth 1996).

Statins have a well established in the prevention of cardiovascular disease and have been shown to exert numerous effects in addition to their lipid-lowering properties. These pleiotropic properties include anti-inflammatory and immunomodulatory effects resulting in improved endothelial function, reduced thrombogenicity and plaque stabilization (Gao et al 2008, Faglas et al 2008, Terblanche et al 2006). Of interest to us is the anti inflammatory property of statins.

Cells	Effect
Endothelial cells	↑eNOS expression and activity ↑Tissue type plasminogen activator (t-PA) expression ↓ROS ↑PPAR-α expression ↓Pro inflammatory cytokine expression ↓MHC class II antigen expression
Smooth Muscle cells	↓Migration and proliferation ↓ROS ↓AT1 receptor expression ↑Apoptosis
Platelets	↓Platelet reactivity ↓ Thromboxane A_2 reactivity
Macrophages	↓ Macrophage growth ↓ MMP expression and secretion ↓iNOS expression ↓Pro inflammatory cytokine expression ↓MCP 1 secretion ↓ IL-8 secretion
Vascular inflammation	↓ High sensitivity c reactive protein level ↓Leucocyte endothelial cell adhesion ↓ ICAM-1 expression ↓ P selectin expression
Other effects	↓ Tumor growth ↓ Intracellular Ca^{2+} mobilization ↑ Bone formation

Table. 6. Effects of statins unrelated to lipid lowering.

Hydroxymethyl-glutaryl (HMG) coenzyme A (CoA) reductase inhibitors or statins exert pleiotropic effects on cellular signalling and cellular functions involved in inflammation (figure 15). Statin-sensitive signalling molecules include Rho guanosine triphosphatases (GTPases), mitogen-activated protein kinases, and Akt (Okuchi et al 2003, Patel and Corbett 2004, Patel and Corbett 2003); statin sensitive cellular functions include adhesion, chemotaxis, and release of superoxide anion (O_2^-) and cytokines (Chello et al 2003, Ando et al 2000, Kanno et al 1999). Although recent reports indicate that some statins may inhibit leukocyte function antigen-1 through direct binding (Weitz-Schmidt et al 2001), it is generally believed that the majority of the observed anti inflammatory effects of statins stem from their inhibiting the prenylation of signalling proteins (e.g., Rho GTPases) (Laufs and Liao 2003). In support of the fact that statins inhibit protein prenylation by depleting the cellular pool of isoprenoids (e.g., geranylgeranyl-pyrophosphate) downstream of mevalonic acid (the product of HMG CoA reductase), reports document that various effects of statins on cellular signalling and functions are blocked by co incubation of cells with mevalonic acid or isoprenoids (Stalker et al 2001, Wong et al 2001, Dunzendorfer et al 2007).

Fessler and colleagues studied the effects of statins in a murine model of LPS induced lung inflammation (Fessler et al 2005). They found that LPS and KC (keratinocyte derived chemokine) induced airway neutrophils were reduced by statins likely arising from an inhibition of the Rho GTPase Rac and actin cytoskeletal remodelling. Statins were also associated with reduced parenchymal MPO and microvascular permeability, and altered airway and serum cytokines after LPS. They concluded that statins attenuate pulmonary inflammation induced by aerosolized LPS and this was dependent on statin inhibition of the mevalonic acid pathway.

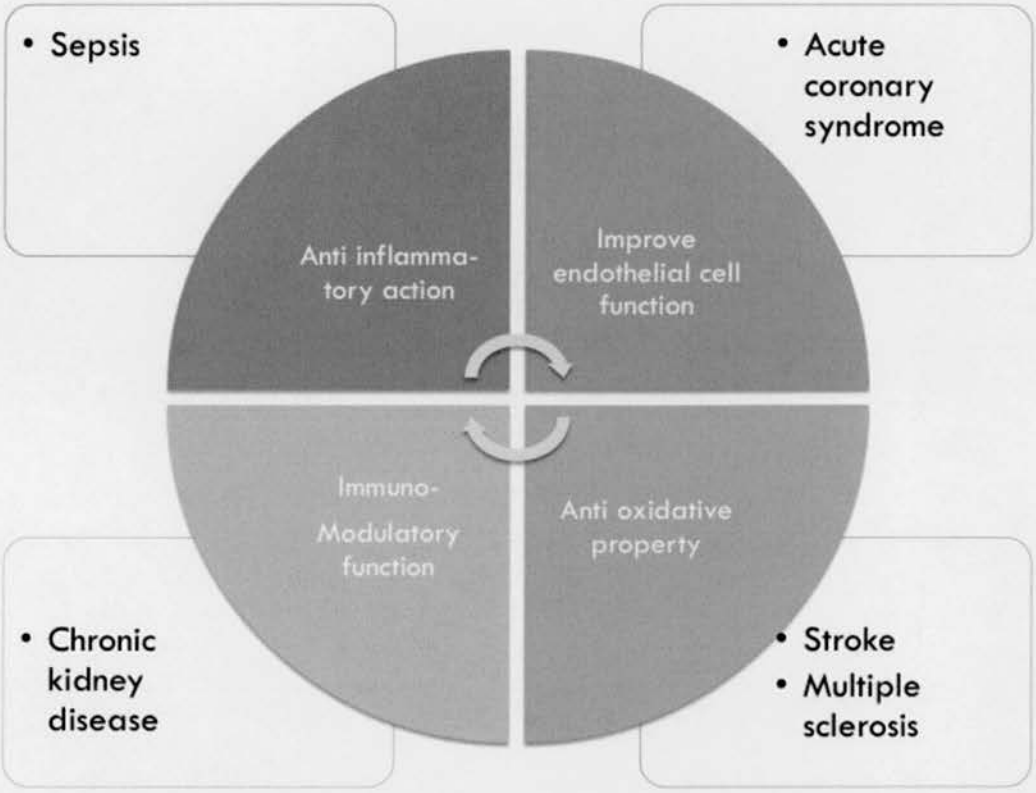


Figure 15. Pleiotropic effects of statins.

1.11.2. STATINS- ROLE IN BACTERIAL INFECTION AND INFLAMMATION

Several studies have reported on the beneficial effect of statins in community acquired pneumonia. In 2004, a prospective observational cohort study of individuals admitted with bacterial infection on statins, had reduced incidence of sepsis and admission to intensive care units (Almog et al 2004). Mortensen *et al* conducted a retrospective study and concluded that prior statin use was associated with reduced 30 day mortality in patients admitted with CAP and sepsis (Mortensen et al 2005, Mortensen et al 2007). Till date, more than 20 studies have reported the effect of statins in CAP and sepsis with a recent meta analysis by Janda *et al*, reporting a strong beneficial effect against pneumonia and sepsis related mortality (Janda et al 2010). However, as there are no RCTs to date that have studied the effects of statins in community acquired pneumonia, the direct evidence of whether statins confer protection in community acquired pneumonia remains to be established.

Several animal studies have been done assessing the effect of statins on bacterial infections. In an animal model of *Staphylococcus aureus*, high dose statin therapy was shown to enhance the formation of extracellular DNA traps by phagocytes in the mice lung and protect against dissemination of infection (Chow et al 2010, Janda et al 2010). Boyd *et al* (Boyd et al 2012), found that mice treated with prolonged high dose statin, (in a model of lung infection with a gram-positive microorganism- *S. pneumoniae,* which produces the cholesterol dependant cytolysin-pneumolysin), had a strong dose dependent effect on protection against *S.pneumoniae* as evidenced by reduced neutrophil infiltration, maintenance of vascular integrity and less chemokine protection. However, mortality was not affected by statin pre-treatment. Antibiotic treatment with ampicillin was initiated 48hours after the microbial challenge and it would be interesting to see if mortality would have been affected if antibiotics were commenced earlier. Another definite caveat to the data on mortality in this study is that rodents metabolize statins at different rates than humans so the beneficial effects in humans could occur in lower doses. While these studies may not directly translate to complex patients settings, it does provide us with extremely important data that might help form the basis for large RCTs in the clinical settings of acute and chronic infection and inflammation.

1.11.3. STATINS IN NORMOCHOLESTERLOEMIC PATIENTS

In a study by Laufs *et al,* normocholesterolaemic men with normal vascular function were treated with high-dose atorvastatin (80 mg). This increased forearm blood flow within 24 h, whereas serum cholesterol and high-sensitivity C reactive protein (hs-CRP) were not decreased until 2 days of treatment. Cessation of statin treatment after 30 days, resulted in a rebound of forearm blood flow within 24 h of withdrawal, whereas cholesterol and hs-CRP slowly and steadily returned to baseline (Laufs et al 2001). This is supported by other studies, involving withdrawal of treatment (e.g., PRISM), where the benefits of statin treatment have been lost sooner than any changes in lipid levels (Heeschen et al 2002, Thomas and Mann 1998). Therefore, the potential importance of cholesterol-independent effects of statins in humans may also relate to the time-course of their effects, for example, after withdrawal of treatment.

1.11.4. WHY ATORVASTATIN?

Formula: $C_{33}H_{35}Ca_2FN_2O_5$

Systematic name: (3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid

Molecular mass: 558.64

First synthesized in 1985 by Bruce Roth, it is a completely synthetic compound.

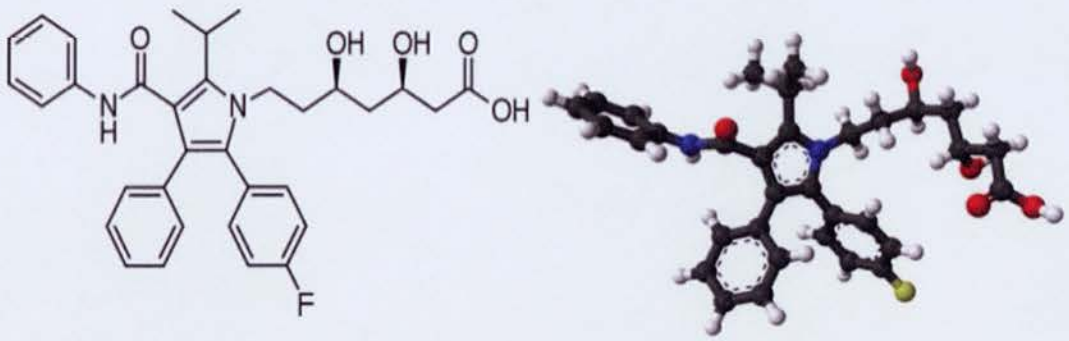


Figure 16. Atorvastatin

Several large clinical trials had identified low-density lipoprotein cholesterol (LDL-C) as one of the major predictors of coronary heart disease (CHD). HMG CoA reductase inhibitors, also known as statins, are considered first-line drugs to prevent CHD because of their potent LDL-C lowering effect (Cannon et al 2004).

1.11.4.1. Comparison of lipid lowering effect

Currently there are six statins commonly used for this indication, including lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, and rosuvastatin. All have a similar therapeutic effect. However, the differences in their chemical structures, pharmacokinetics, and relative efficacy in lipid lowering lead to the question of their

therapeutic equivalence. In a meta analysis conducted by Weng *et al,* where they analysed 75 comparative statin studies, the LDL lowering effect of the different statins. It was determined that atorvastatin 10mg, fluvastatin 80mg, lovastatin 40–80mg, and simvastatin 20mg could decrease LDL-C by 30–40%, and fluvastatin 40mg, lovastatin 10–20mg, pravastatin 20–40mg, and simvastatin 10mg could reduce LDL-C by 20–30%. Rosuvastatin at 10mg or higher dose and atorvastatin at 20mg or higher dose could reduce LDL-C by more than 40% (Weng et al 2010).

1.11.4.2. Comparisons for prevention of coronary heart disease

Till date, there has been two RCTs comparing statins in CHD prevention. Both trials found that atorvastatin 80 mg provided greater protection against unstable angina, in reducing the risk of non-fatal myocardial infarction, peripheral arterial disease and coronary revascularization than pravastatin 40mg or simvastatin 20mg (Cannon et al 2004, Pedersen et al 2005).

1.11.4.3. Comparison of adverse events

The incidence of muscle toxicity was rare in all the included trials. Even when all the muscle related symptoms were considered, the rate was still <10%. However, the studies involving rosuvastatin generally reported a higher adverse event rate than the other comparison arms. When these studies were excluded, the muscle-related adverse events of statins decreased to around 5%. The incidence of elevated ALT / AST level (i.e. three times the upper limit of normal) was <1% in most trials (Weng et al 2010).

In summary atorvastatin is a potent lipid lowering statin, providing greater protection against coronary heart disease, hence ideal for secondary prevention and has a comparatively lower side effect profile. Hence, atorvastatin was the statin of choice in our study.

1.11.4.4. Pharmacokinetics

The systemic bioavailability for HMG CoA reductase activity of atorvastatin is 30%. The primary proposed mechanism of atorvastatin metabolism is through cytochrome P450 3A4 hydroxylation to form active ortho- and parahydroxylated metabolites, as well as various beta-oxidation metabolites. The ortho- and parahydroxylated

metabolites are responsible for 70% of systemic HMG-CoA reductase activity. The ortho-hydroxy metabolite undergoes further metabolism via glucuronidation. As a substrate for the CYP3A4 isozyme, it has shown susceptibility to inhibitors and inducers of CYP3A4 to produce increased or decreased plasma concentrations, respectively. This interaction was tested *in vitro* with concurrent administration of erythromycin, a known CYP3A4 isozyme inhibitor, which resulted in increased plasma concentrations of atorvastatin. It is also an inhibitor of cytochrome 3A4.

Atorvastatin is primarily eliminated via hepatic biliary excretion, with less than 2% recovered in the urine. Atorvastatin has an approximate elimination half-life of 14 h. Noteworthy, the HMG-CoA reductase inhibitory activity appears to have a half-life of 20–30 h, which is thought to be due to the active metabolites.

1.11.4.5. Mechanism of action

As with other statins, atorvastatin is a competitive inhibitor of HMG-CoA reductase. HMG-CoA reductase catalyzes the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate, which is the rate-limiting step in hepatic cholesterol biosynthesis. Inhibition of the enzyme decreases *de novo* cholesterol synthesis, increasing expression of low-density lipoprotein receptors (LDL receptors) on hepatocytes. This increases LDL uptake by the hepatocytes, decreasing the amount of LDL-cholesterol in the blood. Like other statins, atorvastatin also reduces blood levels of triglycerides and slightly increases levels of HDL-cholesterol.

1.11.4.6. Formulations

Atorvastatin calcium tablets are marketed by Pfizer under the trade name of Lipitor, for oral administration. Tablets are available as 10mg, 20mg, 40mg and 80mg and are taken after food at night, to minimize the side effects of leg pain.

1.12. STUDY HYPOTHESIS

Our study hypothesize is that long-term atorvastatin treatment will reduce the persistent neutrophilic airway inflammation in bronchiectasis. This will lead to reduction of patients' symptoms and thereby improve cough, reduce sputum volume and bacterial load, improve airway function and exercise tolerance, reduce exacerbation frequency and consequently improve health related quality of life. Atorvastatin could be a potential novel non antibiotic therapeutic target in bronchiectasis.

This hypothesis then led us to design and conduct a randomized control trial of atorvastatin in bronchiectasis.

1.13. AIMS

The main aims of this randomized double blind control study are:

1. To evaluate the efficacy of a 6 months treatment with atorvastatin versus placebo in patients with clinically significant bronchiectasis.

2. To establish that statin therapy leads to clinical improvements (cough, sputum volume, bacterial load, airway function, exercise tolerance and health related quality of life).

3. To assess if statins reduce neutrophilic airways inflammation by promoting neutrophil apoptosis.

CHAPTER 2

METHODS AND MATERIALS

2.1 METHODS

2.1.1. RANDOMIZED CONTROL TRIAL- STUDY DESIGN AND SETTING

Patients were recruited from South East of Scotland Bronchiectasis Clinic in the Royal Infirmary of Edinburgh, UK from November 2010 to January 2013. Patients were invited to a group meeting and the study was proposed and discussed with them by the research group. Patients were given an opportunity to ask relevant questions regarding the study. They were given up to 7 days for consenting to take part in the study.

Both investigators and participants were blinded to treatment allocation and patients were randomized in equal numbers to receive either atorvastatin 80mg or placebo orally, once daily for 6months.

Sequence generation was done by block randomization of 4 by NHS Tayside, of a list of 60 patients provided by the researchers. The placebo (lactose) was not matched to the atorvastatin in appearance. However the researchers had no access to the study medications as this was provided to the patients directly by pharmacy. We chose atorvastatin as it is a potent statin for reducing cholesterol and we used it at maximum dosage, as this is a proof of concept study.

2.1.2 PARTICIPANTS

Inclusion criteria:

(i) Patients aged 18-79 with an established radiological diagnosis of bronchiectasis (HRCT of the chest) and

(ii) Clinically significant bronchiectasis expectorating mucopurulent or purulent sputum when clinically stable with at least 2 chest infections per year.

Exclusion criteria:

(i) Current smokers or ex-smokers of less than 1 year or those with a greater than fifteen pack year history.

(ii) Patients with cystic fibrosis, active allergic bronchopulmonary aspergillosis, active tuberculosis, poorly controlled asthma, pregnancy or breast feeding,

(iii) known allergy to statins,

(iv) active malignancy,

(v) chronic liver disease,

(vi) statin use in the last year,

(vii) patients on long term oral macrolides due to the interaction with statin therapy,

(viii) patients chronically colonized with *Pseudomonas aeruginosa* (defined as two or more isolates of *Pseudomonas aeruginosa* whilst clinically stable in 6 months prior to the study).

2.1.2.1 *Pseudomonas aeruginosa* colonization

Patients chronically colonized with *Pseudomonas aeruginosa* were excluded, as they are patients with more severe disease (Davies et al 2006, Martinez-Garcia et al 2007) and the objective of this study was to investigate atorvastatin in clinically significant bronchiectasis. Further studies are needed for patients colonized with *P.aeruginosa.*

2.1.3. PRIMARY OUTCOME

The primary outcome of this study was whether there is a difference in the proportion of patients with a clinical improvement in cough [as defined by a 1.3 unit increase in the Leicester Cough Questionnaire[14] score] in those treated with atorvastatin versus placebo. The LCQ has 19 items divided into 3 domains: physical (8 items), psychological (7 items) and social (4 items). The total severity score ranges from 3-21, where a lower score indicating a greater impairment of health status due to cough. We have validated use of this scoring system in bronchiectasis (Murray et al 2009). Cough is a key feature in bronchiectasis and improving this is of major importance to patients with bronchiectasis.

2.1.4. SECONDARY OUTCOMES

Secondary outcomes included: forced expired volume in one second (FEV_1), forced vital capacity (FVC) and its ratio; incremental shuttle walk test; qualitative and quantitative sputum bacteriology; exacerbation frequency; health related quality of life assessed by the St George's Respiratory Questionnaire; assessment of sputum neutrophil numbers and apoptosis; neutrophil activation in the airway by measuring sputum myeloperoxidase and free elastase activity; neutrophil chemottractant in sputum interleukin (IL)-8; systemic inflammation- measuring white cell count, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), additional systemic inflammatory markers [IL-1β, IL-6, IL-8, IL-10, IL-12p70 and tumor necrosis factor (TNF)-α] -and safety of statin therapy.

2.1.5. ASSESSMENTS

Assessments below were done at baseline and 6 months. At 3 months, blood tests and compliance was checked.

Baseline **Start of Study**	**6 months** **Completion of the study**
Cough (Leicester cough questionnaire)	Cough (Leicester cough questionnaire)
Sputum colour	Sputum colour
24 hr sputum volume (ml)	24 hr sputum volume (ml)
Induced Sputum Microbiology	Induced Sputum Microbiology
Bloods FBC, U+E, LFT, CK, ESR,CRP, cholesterol, cytokines	Bloods FBC, U+E, LFT, CK, ESR, CRP, cholesterol, cytokines
FEV_1 + FVC FEV_1/FVC	FEV_1 + FVC FEV_1/FVC
Incremental exercise test	Incremental exercise test
Quality of life questionnaires	Quality of life questionnaires
Induced Sputum MPO, Elastase, IL8 Apoptosis	Induced Sputum MPO, Elastase, IL8 Apoptosis
	Assess side effects and compliance

Table 1. Assessments done at baseline and at end of study.

2.1.5.1. Cough

Assessed using the Leicester Cough Questionnaire (LCQ). It is a 19 item self-completed quality of life measure of chronic cough. It has 3 domains: physical (8 items), psychological (7 items) and social (4 items). The total severity score ranges from 3-21, a lower score indicating greater impairment of health status due to cough. It assesses the impact of symptoms over the preceding 2 weeks and has been used in COPD and asthma. It has been validated for use in bronchiectasis (Murray et al 2009). The LCQ offers a pertinent, timely and useful clinical tool for bronchiectasis. It focuses purely on the impact of cough severity on HRQoL, unlike other questionnaires that encompass multiple respiratory symptoms. Cough is the dominant symptom of bronchiectasis, present in up to 98% of patients and measuring its impact on HRQoL is critical for both disease assessment and targeted management. Additionally, the LCQ is concise, consisting of only 19 items, offering greater patient acceptance than lengthier questionnaires. The minimum clinically important difference for change (MCID) is 1.3 units (Birring et al 2003).

2.1.5.2. Spirometry

Forced expiratory volume in 1 second (FEV_1) and Forced Vital Capacity (FVC) were measured according to standardized guidelines (Miller et al 2005) using a MicroMedical Microloop ML3535 (Viasys Healthcare).

FEV_1 is the maximal volume of air exhaled in the first second of forced expiration following a forced expiration from a position of full inspiration and is expressed in litres. FVC is the maximal volume of air exhaled with maximally forced effort from a maximal inspiration and is expressed in litres. The highest of three technically satisfactory measurements (within 10%) was recorded for each and the ratio was calculated. Actual values were recorded and were also expressed as % predicted for the patients age, sex, and height.

2.1.5.3. Incremental shuttle walk test

The incremental shuttle walk test (ISWT) was used to assess functional exercise capacity. It has been proven in patients with COPD to provide an objective measure of disability and can be used for direct comparison of patients performances (Singh et al 1992).

The ISWT comprises a 10-metre shuttle course with the walking speed externally paced or controlled using pre-recorded audio signals from a computerised disc. It consists of twelve incremental levels, with each level lasting one minute. It is a symptom-limited test with the endpoint of the test determined when the patient becomes too breathless to continue or when the patient becomes unable to complete the shuttle in the paced time (defined as being 0.5metres or further from the marker). All patients were instructed to walk at a steady pace walk aiming to turn around at each end of the course at the sound of the audio signal. All were advised to continue to walk until they felt unable to maintain the required speed without becoming unduly breathless. The level achieved was recorded and total distance covered calculated. At the start and end of the ISWT all patients had their heart rate, oxygen saturations and dysnoea score according to the modified BORG dyspnoea scale (Borg G 1982) in accordance with standardised guidelines.

Modified Borg Score	
0	Nothing at all
0.5	Very very slight (just noticeable)
1	Very slight
2	Slight (light)
3	Moderate
4	Somewhat severe
5	Severe (heavy)
6	
7	Very severe
8 9	
10	Very very severe (almost maximal)

Table 2. Borg score.

Prior to starting the study, all patients attended the physiotherapy gym and or the laboratory to familiarize them with the equipment and to minimize the effects of test habituation. This was done on two separate days to assess reproducibility of tests and this was achieved for all patients.

2.1.5.4. Sputum gram staining

A 10µl sterile loop was used to transfer sputum to a clean, dry, frosted glass slide. 10µl of sterile saline was gently pipetted onto the sputum and the sample smeared uniformly and thinly across the slide. The sample was air-dried and subsequently heat fixed by passing the slide through a low flame several times until all moisture had evaporated. The slide was then flooded with the primary staining reagent methyl violet for one minute followed by gentle rinsing in deionized water. The mordant iodine was then used to flood the slide for one minute followed by further gentle rinsing in deionized water. Following draining, acetone was used as a decolourising agent, flooding the slide until the fluid appeared colourless (approximately ten seconds). The slide was immediately rinsed in deionized water. The counterstain basic fuschin was then used to flood the slide for one minute before further rinsing in deionized water. The slide was gentle blotted using paper towel and allowed to air dry. The results of the staining procedure were observed under oil immersion microscopy. At low (x100) magnification, the number of squamous cells and polymorphonuclear leukocytes per field were counted. At high (x1000) magnification, bacterial cell colour was described as purple (gram positive) or pink (gram negative) and morphology was noted (rods or cocci).

All sputum samples were considered to be valid if there were >25 polymorphonuclear leukocytes and <10squamous cells on gram stain per low-power (x100 magnification) field (Gleckman et al 1988).

2.1.5.5. Cytospins

Induced sputum was washed with 8 times volume of phosphate buffered saline (PBS). This was then centrifuged at 2000g for 10minutes. The supernatant was discarded and then washed with 4 times volume of PBS at 1500g for 10 mins. Supernatant was discarded and then 4 times volume of sputolysin was added and left on roller for 10minutes. Following this, the sputum was filtered through sterile gauze swab and viability was ascertained by exclusion of trypan blue. Viable cells were counted by a hematocytometer. Final centrifuge was done at 2000rpm for 10 minutes at 4°C. After discarding the supernatant, sputum cells were re suspended in PBS. Cytospins were prepared in a Shandon cytospin 4 (Shandon, Pittsburgh, PA, USA) and stained with Diff-Quick stain (Merz Dade, Switzerland). Cell differential counts were determined by counting 400 cells per sample.

2.1.5.6. Quantitative and qualitative sputum microbiology

24 hour sputum volume: Collected the day before each clinic visit. Patients were advised to store sputum samples at 4°C (in the fridge) during collection.

Induced sputum:

Sputum was induced in all study patients at both study visits. Any upper respiratory secretions were cleared before the procedure was commenced. Patient was positioned in a chair to achieve maximum deposition. 30mls hypertonic saline (3%) was used to induce sputum. Most patients were already practicing active cycle breathing techniques (ACBT) or if patients were physiotherapy naïve, the researcher taught ACBT, before commencing procedure. Once commencing the induced sputum procedure, the aerosol was run for 5 minutes and then stopped. At this point the patient was asked to perform ACBT and try to provide a sample. If this is unsuccessful, the whole procedure was repeated again at 5-minute intervals (up to 20 minutes).

Our senior chest physiotherapist advised us that the procedure was to be discontinued if patient started to spontaneously cough or if the patient became distressed, was coughing excessively or saturations dropped below 90%. Sputum induction was successfully carried out in all patients in the study.

Each sample was confirmed to be a valid sample suitable for processing if there are >25 polymorphonuclear leukocytes and <10 squamous cells present on Gram stain on low power magnification. 1 ml of the sample was used for qualitative and quantitative microbiology.

Sputum colour: Graded as mucoid, mucopurulent or purulent (Murray et al 2009).

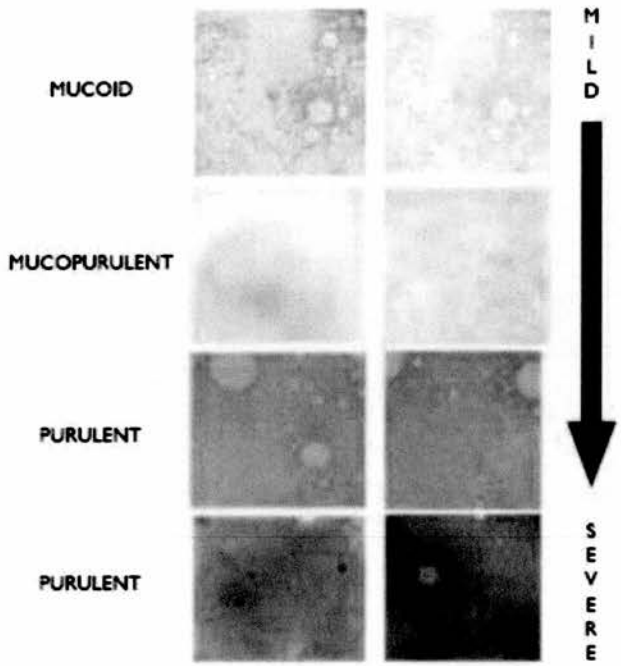


Figure 1. Sputum colour chart (Murray *et al* 2009)

2.1.5.7. Sputum processing

Sputum was homogenized and liquefied using an equal volume of dithiothreitol and serially diluted using sterile 0.85% saline to achieve dilutional factors of 10^{-1} to 10^{-4}. *Pseudomonas* isolation agar (Difco), chocolate blood agar containing bacitracin (Oxoid) and horse blood agar (Oxoid) plates were inoculated with 100 µl of dilution. These were incubated at 37°C for 48 hrs. Colonies of the pathogens were then counted to determine the sputum bacterial density, expressed as $\log_{10}$ colony forming units/ml ($cfu.ml^{-1}$). The rest of the sample was divided equally into portions; the first was ultracentrifuged at 30000g for 90 min at 4°C (Hill et al 1999).The sol phase was stored at -70°C until needed for analyzing.

2.1.5.8. Qualitative sputum bacteriology

Bacterial Count was calculated: Number of bacteria x 2 x 10 x dilution factor to give the number of cfu/ml.

Viable pathogens were identified following incubation based on colonial morphology, Gram stain and further, specific standardised identification tests. The identification methods used for the most common organisms cultured is described.

2.1.5.9. *Haemophilus influenzae*

The primary isolation media used for *Haemophilus influenzae* was chocolate with bacitracin agar incubated at 37°C at 5%CO. Colonies are small, round and convex and typically appear after 24hrs incubation. The species appear negative on Gram staining as spherical, oval or rod shaped cells of less than 1µm diameter. Colonies suspected to be *Haemophilus* species from morphology and Gram stain appearance were further identified based on their requirement for X and V factors. One or more colonies were selected using a sterile straight wire loop and emulsified in distilled water to produce a light suspension. 100µl of suspension was inoculated onto nutrient agar and spread evenly using a sterile hockey stick spreader. Three filter paper discs incorporating X factor (comprising protoporphyrin IX and haemin), V factor (comprising nicotinamide adenine dinucleotide) and XV factors together respectively, were positioned on the inoculated agar surface in the configuration of an equilateral triangle with a minimum of 3.5cm distance between the discs. Following overnight incubation at 37°C, the agar plate was examined for growth around the discs.

Haemophilus influenzae was distinguished from *Haemophilus parainfluenzae* as detailed in Table 3.

Factor	***Haemophilus influenzae***	***Haemophilus parainfluenzae***
X	Negative for growth around disc	Negative for growth around disc
V	Negative for growth around disc	Positive for growth around disc
X and V	Positive for growth around disc	Positive for growth around disc

Table 3. X, V and XV test results for *Haemophilus influenzae* and *Haemophilus parainfluenzae.*

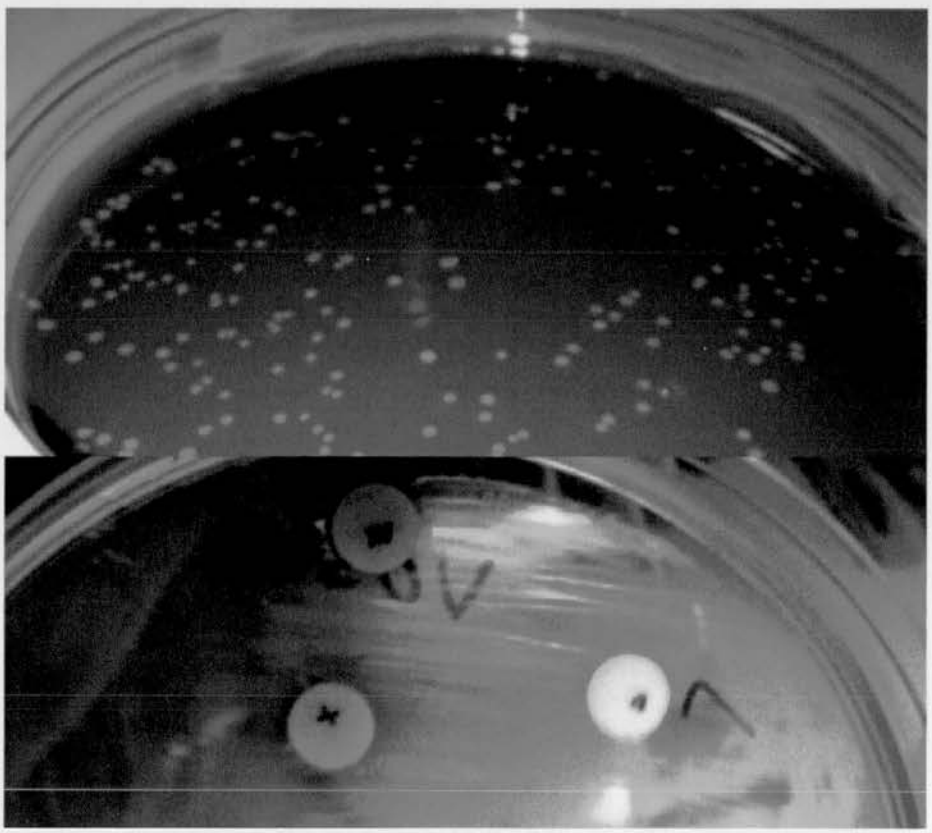


Figure 2. *Haemophilus influenzae* isolated from sputum in our laboratory (upper panel) and the XV tests (lower panel).

2.1.5.10. *Streptococcus pneumoniae*

The primary isolation media used for *Streptococcus* species was horse blood agar. Colonies appear white, 1-2mm in diameter and may have a classic draughtsman appearance due to autolysis after incubation. Streptococcus species are positive on Gram staining, appear round and are in pairs, chains or clusters. Colonies suspected from morphology and Gram stain appearance to be *Streptococcus pneumoniae* was further identified by assessing their sensitivity to ethylhydrocupreine hydrochloride (optochin test). Ethylhydrocupreine hydrochloride causes changes in surface tension of the cell membrane causing *Streptococcus pneumoniae* to lyse. Fresh suspicious colonies were selected using a sterile straight wire loop and streaked across a horse blood agar plate. An optochin disc (filter paper disc impregnated with 5μg of ethylhydrocupreine hydrochloride) was placed in the centre of the inoculated agar and incubated at 37°C at 5% CO for 24hours. Following incubation, the inoculated agar was examined for zones of inhibition. A positive result was defined as a radial zone of inhibition measuring 5mm or more from the edge of the disc. A negative result was defined as either no zone of inhibition or a zone of inhibition less than 5mm radius from the edge of the disc.

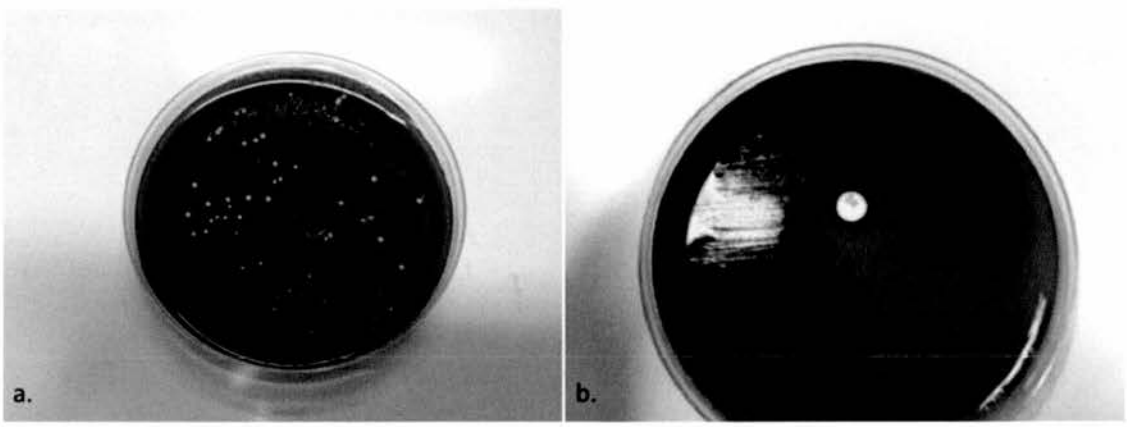


Figure 3a. *S. pneumoniae*. b. Optochin disc test confirming *S pneumoniae.*

2.1.5.11. *Staphylococcus aureus*

The primary isolation media used for *Staphylococcus aureus* was horse blood agar. Colonies are opaque with either a creamy white colour or a yellow-orange colour. Gram staining shows gram positive cocci occurring either singly, in pairs or in irregular clusters. Colonies suspected to be Staphylococcus aureus were confirmed using the commercial test kit Dryspot Staphytect Plus, a latex slide agglutination test according to the manufacturer's instructions (Oxoid Limited, Basingstoke, Hampshire).

Figure 4. *Staphylococcus aureus* isolated in our laboratory.

2.1.5.12. *Pseudomonas aeruginosa*

Cetrimide (Difco) Pseudomonas isolation agar was used as the primary selective media for *Pseudomonas aeruginosa*. Morphological identification of colonies may have included several characteristics. The most common type of colony is a large, low oval convex shape with a rough appearance. There may be a characteristic smell of aminoacetophenone and colonies may have a blue-green appearance due to the production of pyocyanin (blue) and pyoverdin (yellow). The production of this pigment is indicative of *Pseudomonas aeruginosa*, although some strains particularly mucoid strains may not produce pyocyanin. Further identification of colonies was confirmed using the commercially available API20NE kit (bioMerieux UK Linited, Basingstoke, Hampshire) according to the manufacturers' instructions. Briefly, approximately one to four colonies were selected using a sterile straight wire loop and emulsified with 2ml of 0.85% sterile saline to produce a suspension approximating a 0.5McFarland standard. The API20NE test strip consists of twenty microtubes containing dehydrated substances. These microtubes were inoculated with the bacterial suspension and incubated for 24 or 48 hours at 30°C with the addition of reagants and interpretation of reactions done according to the manufacturer's directions. The biochemical reactions were converted accordingly into an octal profile number and decoded using the Analytical Profile Index (API Database Vn6.0, APILAB Software Vn3.3.3, Apilab Plus; bioMerieux).

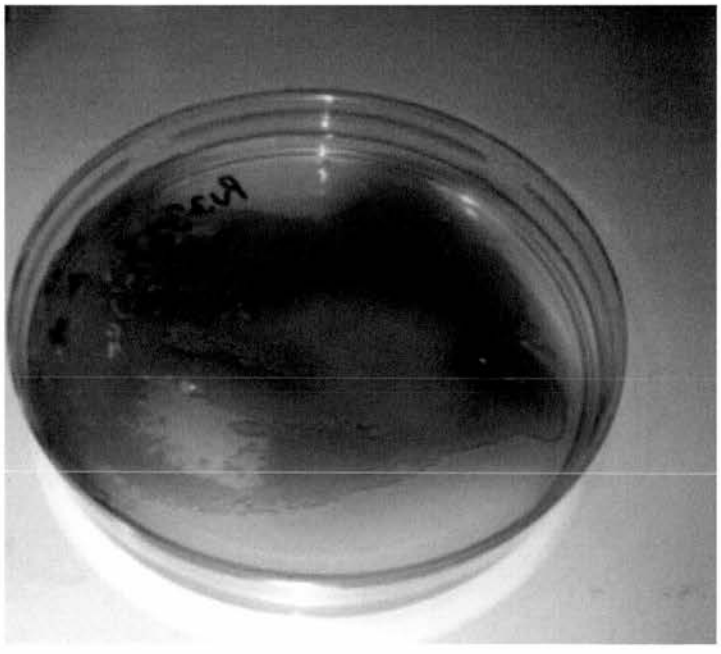


Figure 5. Mucoid *P. aeruginosa* isolated in our laboratory.

2.1.5.13. *Moraxella catarrhalis*

The primary isolation media used for *Moraxella* species was horse blood agar. Colonies are white or buff and convex in shape. The species appear as negative cocci on Gram stain, are approximately 0.6-1.0μm diameter, and occur either singly or in pairs. Colonies suspected to be *Moraxella* species from morphology and Gram stain appearance were tested for a positive oxidase reaction. Filter paper was soaked in the test reagent N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride. A sterile wooden stick was used to select at least one suspicious colony and rub it onto the pre-soaked filter paper. A reaction was looked for within ten seconds. A positive result was confirmed with the development of a blue colour, indicating oxidase production. A negative result was indicated by an absence of colour.

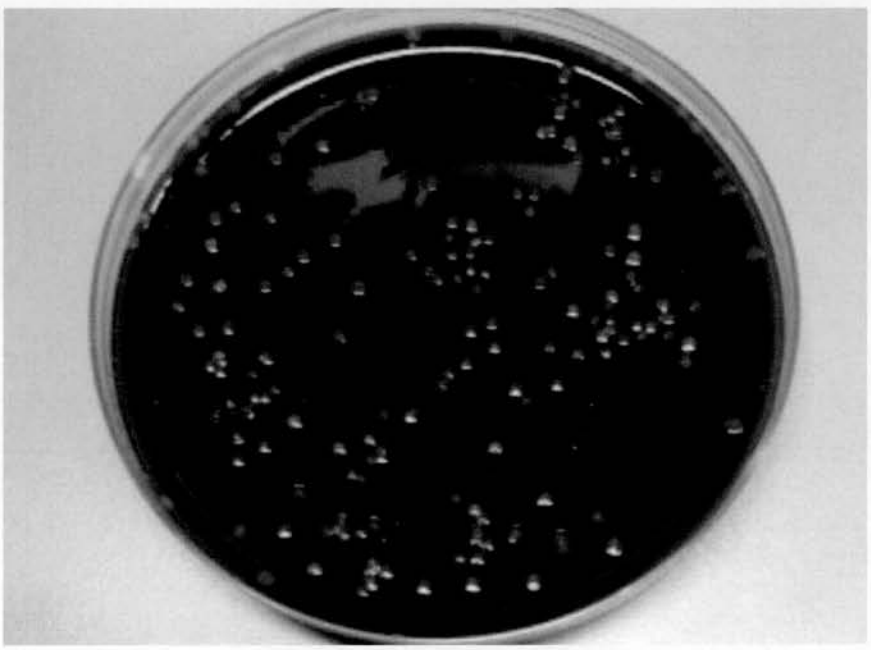


Figure 6. *M. catarrhalis* isolated in our laboratory.

2.1.5.14. *Enterobacteriaceae*

Enterobacteriaceae were selected from horse blood agar. The colonies are 2-3mm in diameter, are low, convex, grey and maybe smooth or mucoid. The cells appear as Gram negative rods. Further identification of suspicious colonies was initially with a negative oxidase test as described previously and then the commercial kit API20E was used (bioMerieux UK Linited, Basingstoke, Hampshire). The principle and preparation of the API20E strip is similar to the API20NE strip described above, with incubation for only 24 hours at 30°C.

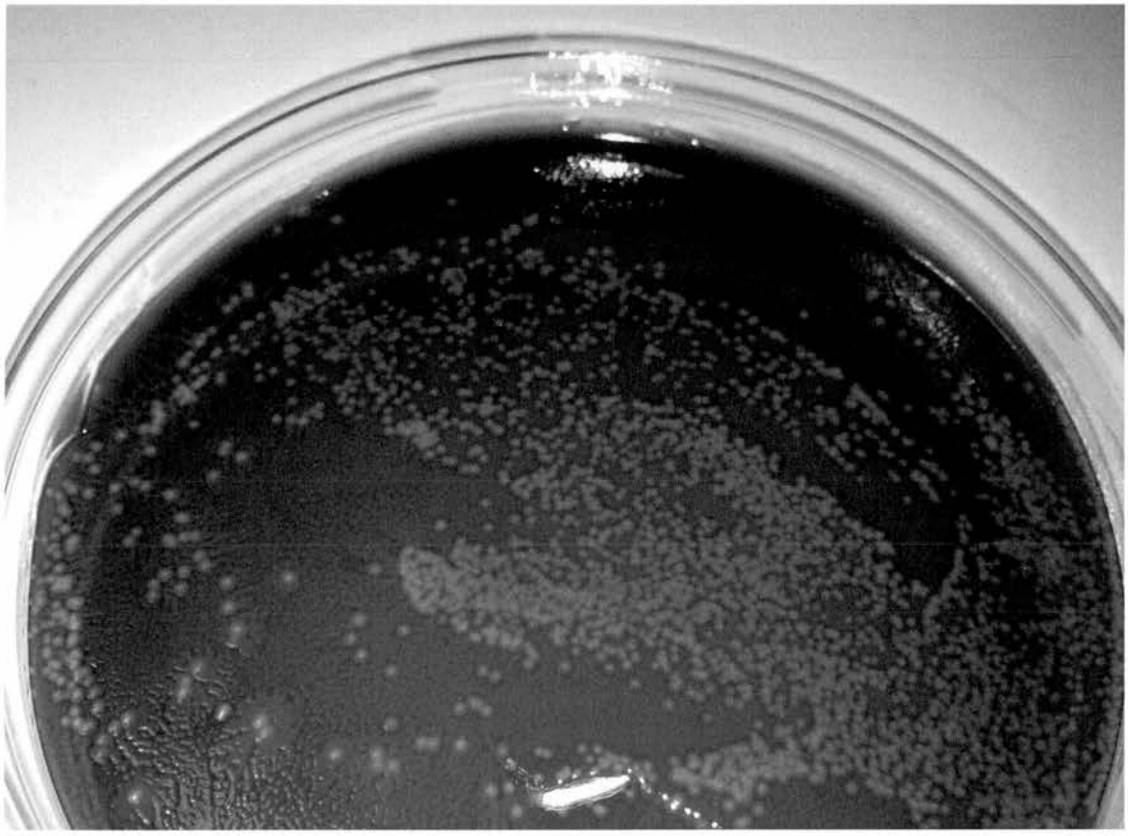


Figure 7. Gram negative microorganism isolated from sputum of bronchiectasis patient in our laboratory.

2.1.5.15. Sputum inflammation markers

For measurement of markers of airway inflammation, sputum, not treated with DTT, was ultracentrifuged (Sorvall™ Ultracentrifuge, UK) at 30000g for 90 minutes at 4°C. The sol phase was removed, immediately frozen in aliquots at -80°C. We measured sputum Myeloperoxidase, free neutrophil elastase and IL-8.

2.1.5.15.1. Myeloperoxidase (MPO): MPO activity was measured by a chromogenic substrate assay.

Myeloperoxidase (Calbiochem ®) and samples were diluted as necessary in phosphate buffered solution. All reagents were brought to room temperature first. 25μL of standard or sample was added to the wells of a 96 well microtitre plate (Costar®). 25μL of tetramethylbenzidine (Sigma) was added to each well. The plate was then incubated for 5minutes at 25°C. Reaction was stopped by adding 50uL of sulphuric acid solution to each well. Absorbance was measured using a dual wavelength of 450 and 560nm and MPO concentration interpolated from the standard curve and expressed as μg/ml. The MPO concentration was determined in duplicate for each sample or standard and the mean determined for each.

2.1.5.15.2. Free Elastase activity (NE):

NE activity present in the samples was measured spectrophotometrically using the synthetic substrate methoxysuccinyl-ala-ala-pro-valparanitroanilide [MeOSAAPVpNa 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid] (Sigma) as described below (Hill et al 1999, Stockley et al 2000).

Standards (NE from Sigma Aldrich) and samples were diluted as necessary in buffer. 40μL of standard or sample were added to each well of a 96 well microtitre plate (Costar®). 40μl of MeoSAAPvn was added to each well and samples read immediately at 37°C for a minimum of thirty minutes with readings every two minutes.

The rate of change in optical density is converted into elastase activity and expressed in units per milligram. The elastase concentration for each sample is determined in duplicate and the mean determined for each.

2.1.5.15.3. Measurement of IL-8:

IL-8 is a key neutrophil chemoatttractant in bronchiectasis (Milkami et al 1998). Untreated sputum sol phase was assayed using commercially available specific enzyme linked immunosorbant assay (ELISA, R+D systems, Abingdon, UK) using kits previously validated for sputum use according to established methodology (Stockley and Bayley 2000).

2.1.5.16. Validation of sputum ELISA's

All assays for sputum were validated as described (Stockley and Bayley 2000). Standard curves for each assay were obtained using pure mediator provided by the manufacturer following the assay protocol. Three pools of sputum sol were prepared from 4 patients each with mucoid (grade 1), muco-purulent (grade 2) and purulent sputum (grade 3 or 4) based on a previously published sputum colour chart (Murray et al 2009). Three parameters were assessed in validating assays: Reliability, recovery of spiked mediators and the effect of sample dilution.

Reliability: The quantity of mediator in each sputum pool was determined by interpolation against the standard curve. Individual samples were assayed 5 times on a single plate to obtain the intraassay co-efficient of variation. Each sample was also assayed 5 times on different plates to obtain the inter-assay co-efficient of variation.

Spike and recovery: a known quantity of each mediator was spiked into the 3 pools of sputum. These "spiked" samples were then assayed and compared to the values obtained for the original pool. The obtained value was divided by the predicted value to calculate the % recovery.
Dilution effect: The 3 sputum pools were assayed at dilutions ranging from 1 in 1000, to 1 in 5.

An assay was deemed to be valid if it had a reliable standard curve, an intra-assay and inter- assay coefficient of variation <10%, recovery of spiked samples in the range 80-120% of predicted and a linear dilution effect (Stockley and Bayley 2000). The results of Interleukin-8 assay is shown below, figure 8. This assay passed each validation step and was used in the study. Table 4 shows the validation data for all sputum assays tested.

The figure below shows the validation data for IL-8. Figure 9 shows the linearity of dilution effect. In view of these results, dilution of 1 in 20 was used for each assay with re-assay at higher or lower dilution in cases where results were outside the dynamic range of the assay.

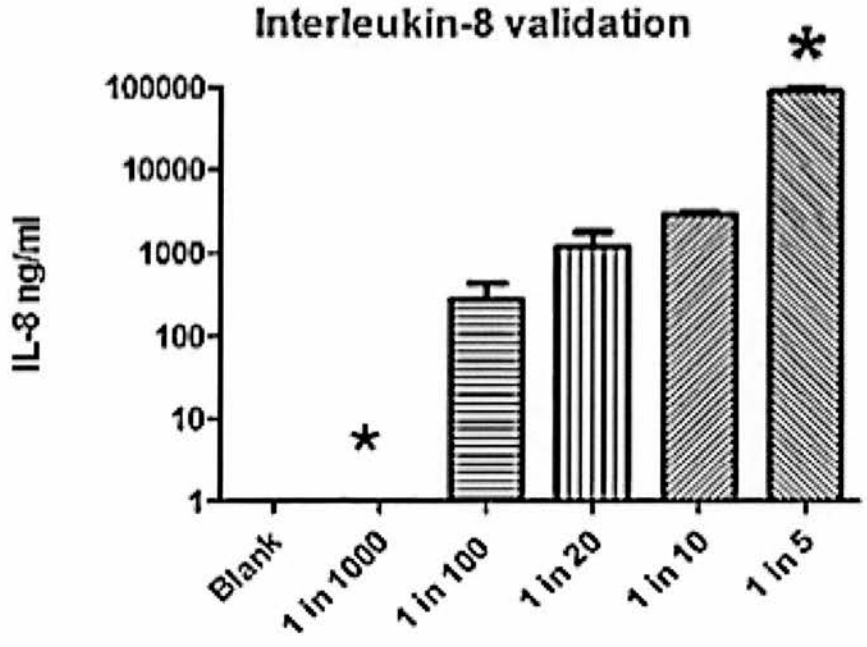


Figure 8. Validation of assays for sputum cytokine measurements: linearity of dilution effects for sputum measurement of interleukin-8. * indicates outside the dynamic range of the assay.

Each assay gave a linear, reliable standard curve and representative standard curves are shown below.

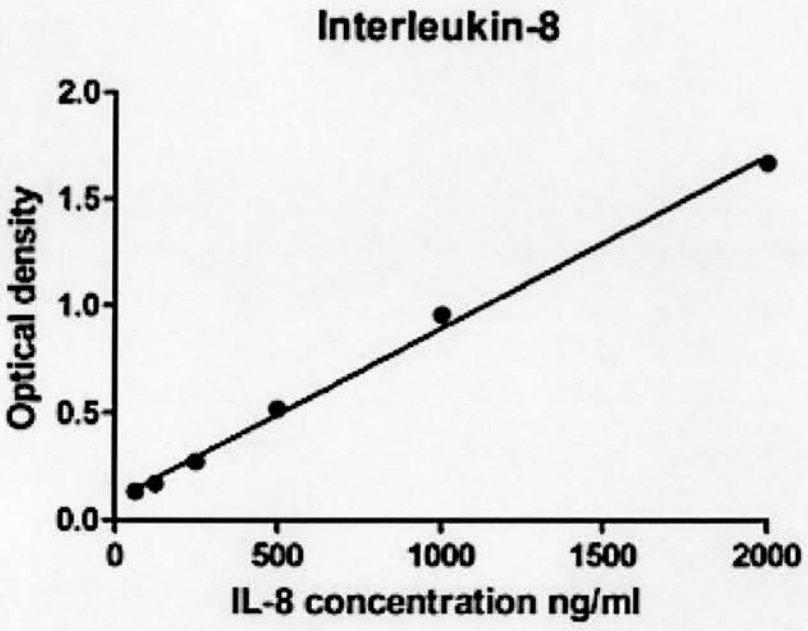


Figure 9. Validation of sputum assays for inflammatory mediators: representative standard curve for sputum measurement of interleukin-8.

Finally, spike a recovery experiments demonstrated a majority of values between 80% and 120% of expected recovery with a median recovery and range within acceptable levels of variability. The overall assessment of each assay tested is shown in table 4.

Mediator	Spike-recovery (median %-IQR)	Intra-assay co- efficient	Inter-assay co- efficient	Linear dilution effect
Myeloperoxidase	102% (94-105)	4.2%	3.7%	Yes
Free neutrophil elastase	102% (83-115)	7.1%	7.7%	Yes
Interleukin-8	93% (86-118)	8.5%	8.1%	Yes

Table 4. Validation of assays used in sputum

2.1.5.17. Health related quality of life- St. George's Respiratory Questionnaire

Health related quality of life was measured using the St George's Respiratory Questionnaire (Wilson et al 1997). It is a 50 item self administered health related quality of life questionnaire divided into 3 main domains, symptoms, activities and impacts. The total score ranges from 0-100; a higher score indicates a poorer health related quality of life. The MCID for SGRQ is 4 units (Wilson et al 1997).

2.1.5.18. Systemic markers of inflammation

30 ml of venous blood was collected for full blood count, ESR, CRP, urea, electrolytes, creatinine kinase, liver function tests and chloesterol.

5 ml of blood was centrifuged at 750g for 10 min and the supernatant collected and stored at -70C, for measuring pro and anti inflammatory cytokines and chemoattractants by cytometric bead array (BD™ Cytometric Bead Array kits). Cytometric bead array was done as per manufacturer' instructions (preconfigured kits for consistent results and an intra-assay and inter- assay coefficient of variation <10%, recovery of spiked samples in the range 86-110% of predicted and a linear dilution effect) and we measured IL1β, IL6, IL-8, IL10, IL12p70 and TNFα.

2.1.6. SIDE EFFECTS

The presence or absence of side effects was assessed at all study visits. If the alanine aminotransaminase was greater than five times normal or the creatinine kinase greater than three times normal, the study medication was stopped. All side effects were recorded in a patient diary card (Appendix 1).

2.1.7. INFECTIVE EXACERBATIONS DURING THE STUDY

Exacerbations were defined as per the BTS guidelines (BTS guidelines 2010). Exacerbations were treated according to their baseline sputum bacteriology and received 14 days oral antibiotic treatment. Macrolides were not used because of the interaction with statin therapy.

2.1.8. SAMPLE SIZE

Using a two-sided, two-sample test with a 5% level of significance, 90% power, a sample size per group of 27 was needed to detect a change of 1.3 Units in the Leicester Cough Questionnaire. 30 patients were recruited in each group to allow for dropouts,

2.1.9. STATISTICAL ANALYSIS

All data was analyzed on SAS Version 9.2. We used an intention to treat analysis for the primary endpoint and a modified intention to treat analysis for the secondary endpoints. For demographic and clinical variables, data are presented as median (interquartile range) for continuous variables and n (%) for categorical variables unless otherwise stated. Baseline to 6 month change of LCQ was calculated by an unpaired t-test between those receiving atorvastatin versus placebo, as the data was normally distributed. To compare the proportion of patients with clinical improvement as measured by the LCQ or quality of life as measured by the SGRQ a binomial test for the comparison of proportions has been used and differences presented as percentages with accompanying 95% confidence intervals [CI]. Categorical data have been compared between groups using Chi-squared test. A P-value of <0.05 was considered statistically significant for each analysis.

2.2. NEUTROPHIL ISOLATION

Ethical approval was obtained from the Lothian Research Ethics Committee (Approval #08/S1103/38).

Requirements:

- Sodium citrate 3.8 % (3.8 g Sodium citrate tribasic dihydrate (SIGMA 25116) in 100 ml bottled water (BAXTER UKF7114)) (solution is sterile filtered using a 0.22 μm filter unit)
- 6% Dextran [6.0g Dextran 500 (GE Healthcare 17-0320-02*) in 100ml 0.9% NaCl (BAXTER UKF7124)]. Dissolve in 0.9% NaCl Saline warmed to 37°C (solution is sterile filtered using a 0.22 μm filter unit)
- Percoll (GE healthcare 17-0891-02)
- PBS (phosphate buffered saline) – without Ca^{2+} /Mg^{2+} (PAA H15-002)
- PBS (x10) - without Ca^{2+} /Mg^{2+} (Sigma D1408)
- Iscove's modified Dulbecco's modified Eagle's medium (IMDM) (PAA E15-018)

Method:

All procedures carried out at room temperature unless otherwise stated.

Freshly drawn blood was collected from healthy volunteers, into sodium citrate; (4ml of citrate per 40 ml of blood in a 50 ml falcon tube) and mixed by gentle inversion of tube, parafilm cap before centrifugation. This was centrifuged at 350g for 20 minutes (Acc1/ Brake 0 Hettich centrifuge, Acc0/Brake 0 Mistral centrifuge).

Platelet-rich plasma (PRP) was aspirated without disturbing the pelleted cells. Autologous recalcified plasma (serum) was prepared by adding 220 μl of 1M CaCl /10 ml plasma in glass tubes at 37°C for 1 hour. "Serum" was separated from platelet plug and transferred to a Falcon tube for later use/storage (4°C for short term or frozen for longer term storage). Leukocytes were separated from erythrocytes by dextran sedimentation: 6 ml of dextran (see above) was added to each tube (adjust volume of dextran added if necessary – 2.5ml / 10ml cell pellet) and then made to 50ml with saline pre-warmed to 37°C. This was then mixed carefully to ensure cells

were fully resuspended then allowed to sediment for between 20 and 30 mins (not more than 30 minutes) at room temperature.

Percoll gradients were prepared at this point-at room temp. Stock Percoll solution was made isotonic with 10x PBS (WITHOUT Ca^{2+}/Mg^{2+}) (yields a 90% solution that represents "stock")(enough for 3 grads- i.e. 6 tubes of blood: 27 ml Percoll + 3ml 10xPBS)

81% Percoll made in PBS WITHOUT Ca^{2+}/Mg^{2+} (8.1ml Percoll "stock" +1.9 ml PBS)

70% Percoll made in PBS WITHOUT Ca^{2+}/Mg^{2+} (7ml Percoll "stock" + 3 ml PBS)

55% Percoll made in PBS WITHOUT Ca^{2+}/Mg^{2+} (5.5ml Percoll "stock" + 4.5ml PBS).

Following dextran sedimentation, each leukocyte-rich upper layer was removed and transferred to a fresh 50 ml tube and topped up to 50ml with saline and then centrifuged at 350g for 6 min (Acc5/ Brake 5 for both Hettich and Mistral centrifuges).

The pellets from 2 tubes of cells were resuspended in 3ml of the 55% Percoll (upper layer).

3ml of 81% Percoll (bottom layer) was carefully placed in the bottom of a 15ml Falcon tube.

3ml of 70% Percoll (middle layer) was carefully overlayered onto the bottom layer (slowly to avoid mixing of the gradients).

3ml of cells resuspended in the 55% Percoll layer was carefully overlayered onto the middle layer.

Gradients were centrifuged at 720g for 20 min, (Acc1/ Brake 0 Hettich centrifuge, Acc0/Brake 0 Mistral centrifuge).

Granulocytes were harvested (70/81 interface) and residual erythrocytes pelleted at the bottom of the tube.

Leukocytes were then washed twice in PBS without Ca^{2+}/Mg^{2+} centrifuged at 230g for 6 min, (Acc5/ Brake5 Hettich and Mistral centrifuges). Cells were resuspended at desired concentrations in media and counted using a haemocytometer. 10µl of the granulocyte suspension was placed on the haemocytometer slide and visualised under a light microscope, the number of neutrophils present in 25 squares were counted. The

number obtained was equal to the number of neutrophils per 0.1µl of suspension, to calculate the number of neutrophils in 1ml, this was multiplied by 10,000.

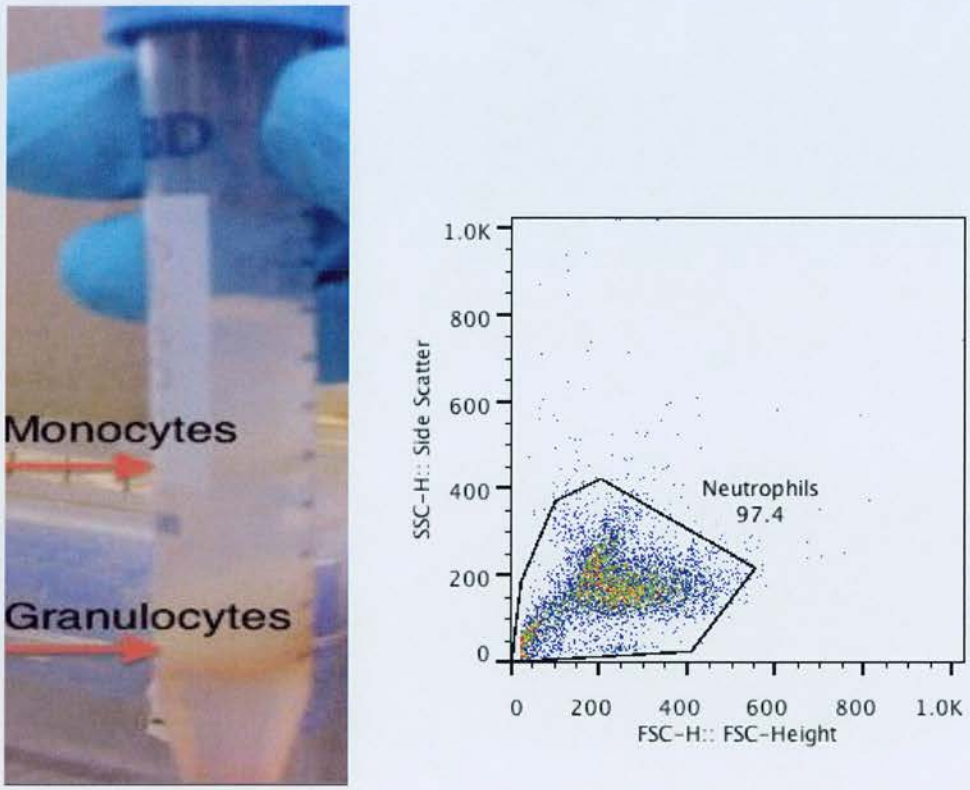


Figure 9. Neutrophil isolation by Percoll gradient (left). Isolated neutrophils on flow plot (right).

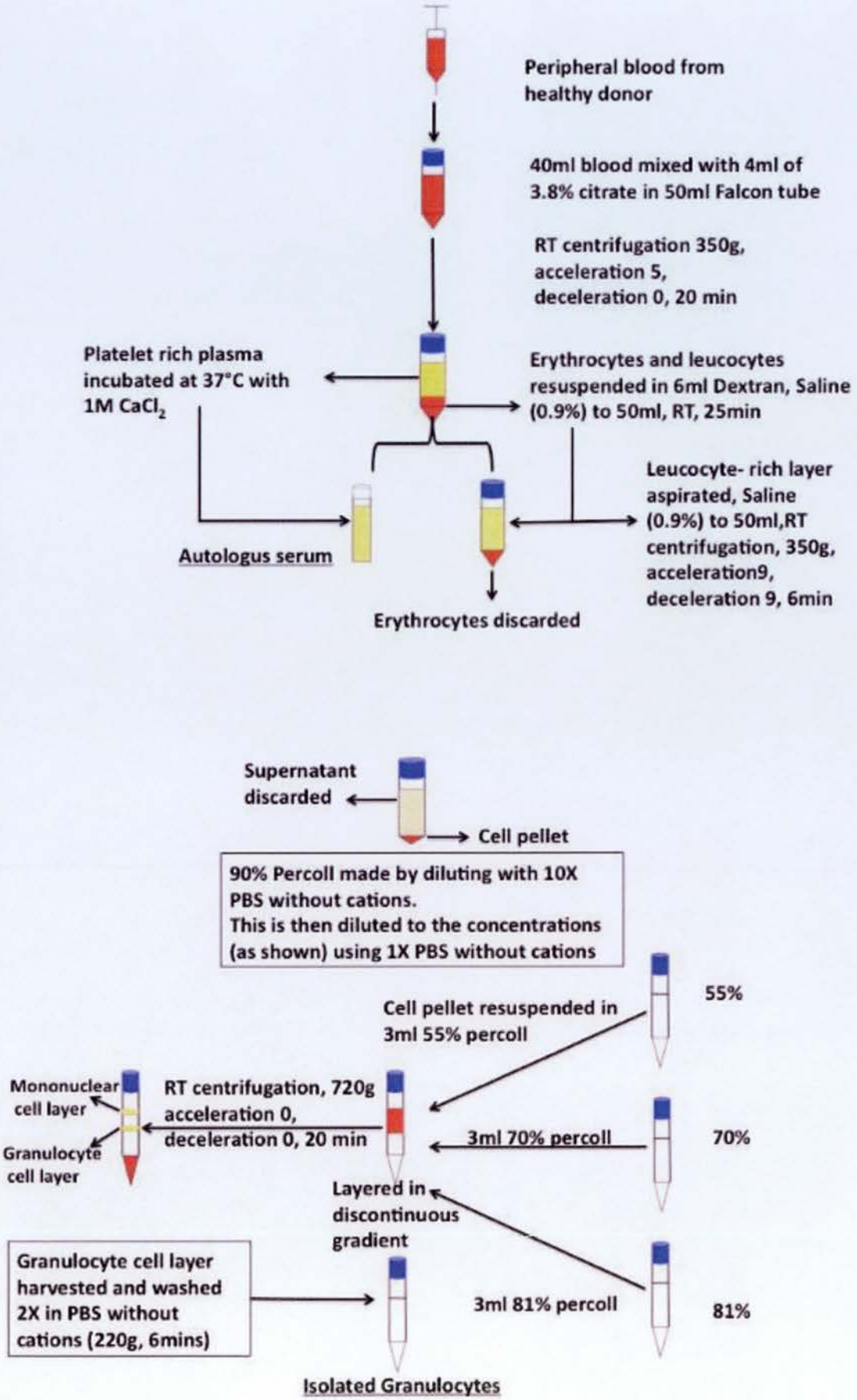


Figure 10. Isolation of neutrophils by Percoll gradient.

2.3. APOPTOSIS ASSAY

Freshly isolated peripheral blood neutrophils (at least 97% purity – performed cytocentrifuge preparation as described above) was suspended at $10x10^6$ cells/ml in IMDM supplemented with 10% autologous serum and penicillin/streptomycin (1x). Then 75 µl of neutrophil suspension was added to wells of a 96 well flat-bottom plate. A row of wells was left clear from the periphery of the plate- to allow for addition of reaction media to minimise evaporation. To each well 15µl of treatment (10 times concentration) or 20µM of roscovitine or buffer control was added. Following this, 60ml IMDM with 10% serum was added to each well. If two agents were used in combination only 45ml of IMDM was required. Each treatment was done in duplicates or triplicates. Plates were then covered with a lid, and incubated at 37°C in a 5% CO_2 incubator for the desired length of time (12 or 20 hours).

Dose and time response experiments were done.

Following incubation for the desired length of time, the plates were taken out of the incubator. Each well was then vigorously pipetted to dislodge adherent cells and transfer 20µl of cells into a flow tube containing 200µl of Annexin V buffer [Annexin buffer= annexin V (Roche, West Sussex, UK)+ HBBS with Ca and Mg in the ratio of 1:500]. The flow tubes were then incubated for 5 min on ice. Immediately prior to running each sample on a flow cytometer propidium iodide (Sigma- Aldrich) (PI; 1µl of 1mg/ml stock solution) was added. Samples were analysed by flow cytometry using FL-1/FL-2 channel analysis following appropriate compensation. Cytospins were done for each of the different treatments.

CHAPTER 3

RESULTS

3.1. Completion/ Timing of visit

A total of 62 patients were recruited into this study, 2 were lost prior to randomization so were replaced to give a total sample size of 60 patients. Through the course of the study 7 patients withdrew and the remaining 53 (83%) patients completed the study. 24 (80%) patients completed in the statin group and 29 (97%) in the placebo group (see consort diagram).

The following table shows the timing of visit in only the 53 participants who completed the study.

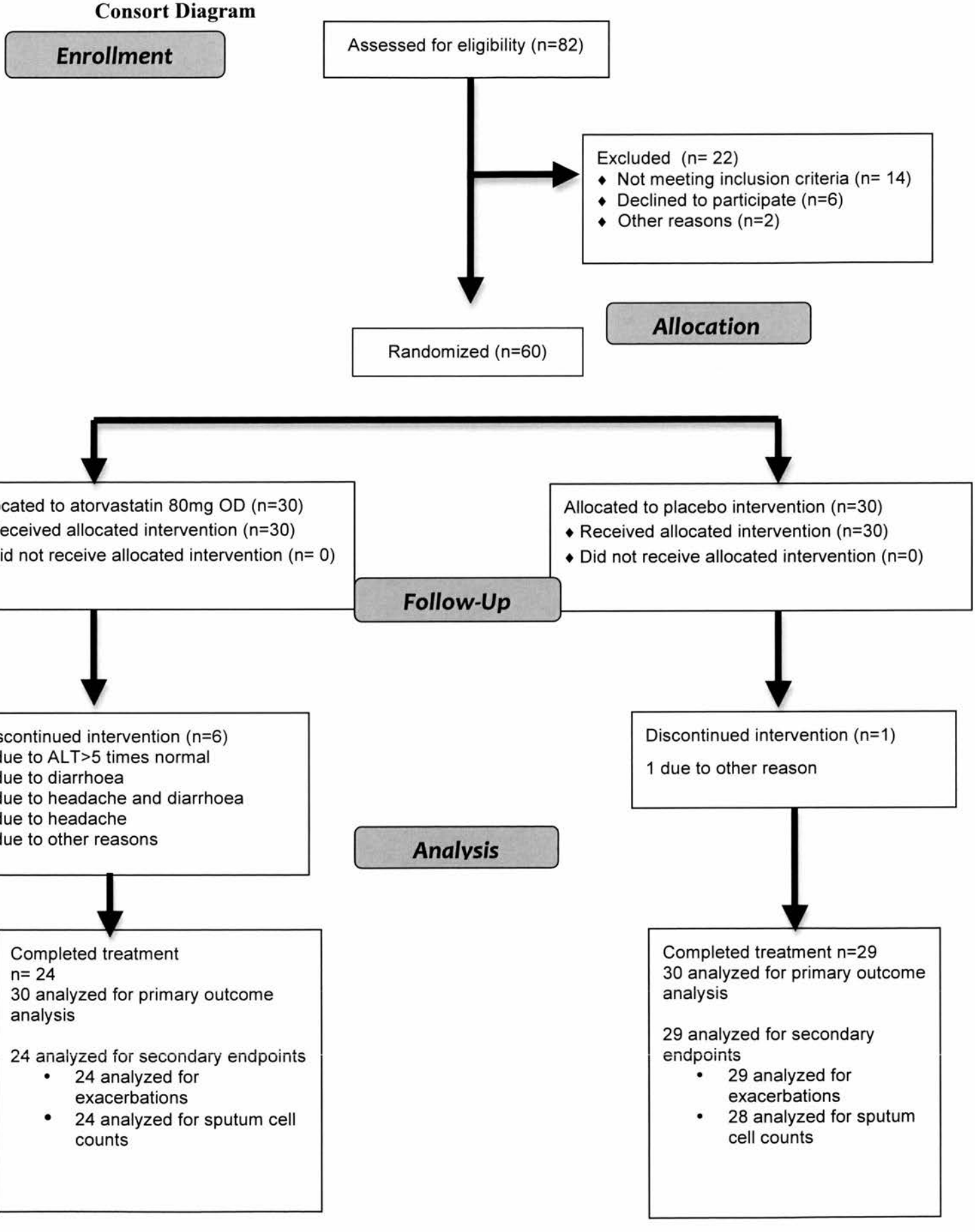
Randomized treatment	N	Min.	Lower quartile	Median	Upper Quartile	Max.
Atorvastatin	24	176.0	180.5	183.5	191.0	211.0
Placebo	29	175.0	182.0	184.0	191.0	206.0

Table 1. Timing of 6month visit in patients completing the study.

3.2. Baseline Characteristics

60 patients were randomized to receive treatment; 30 received active treatment with atorvastatin 80 mg and 30 received placebo. Baseline characteristics of the study population were not statistically significant different between the two groups (see table 2) with the exception of smoking. The smoking differences were not clinically relevant as we excluded current smokers, smokers >15 pack year history and those with COPD. 2 patients in the statin group isolated *Pseudomonas aeruginosa* at baseline only and were not chronically infected with *Pseudomonas aeruginosa* as per inclusion and exclusion criteria.

Consort Diagram



	Group receiving atorvastatin 80mg Mean (SD) N=30	Group receiving placebo Mean (SD) N=30	P value
Age	60.2 (10.7)	59.1 (11.4)	0.719
Gender (% female)	17 (57%)	14 (47%)	0.438
Smoking status			
Never	26 (87%)	18 (60%)	0.039*
Ex	4 (13%)	12 (40%)	
BMI (Kg/m^2)	28.8 (8)	28.1 (6.3)	0.709
Comorbidities			
High BMI >30Kg/m^2	11 (37%)	10 (33%)	0.787
IHD	2 (7%)	1 (3%)	1.00*
Asthma	19 (63%)	17 (57%)	0.598
Previous malignancy	1 (3%)	1 (3%)	1.00*
Diabetes mellitus	1 (3%)	1 (3%)	1.00*
Pulmonary physiology			
Pre therapy FEV_1 (L)	2.1 (0.8)	2.2 (0.9)	0.457
FEV_1 (% predicted)	78.3% (23.8%)	73.9% (24.5%)	0.488
Pre therapy FVC (L)	3 (1.1)	3.2 (1.1)	0.494
FVC (%predicted)	94.8% (4.2%)	86.4% (25.6%)	0.190
FEV_1 /FVC	69% (0.13%)	69.6% (0.12%)	
Inflammatory markers in serum			
White cell count	7.2 (2.2)	6.7 (1.9)	0.341

(X10^9/L)			
Neutrophils (X10^9/L)	4.3 (1.9)	3.9 (1.1)	0.280
ESR (mm/hr)	15.1 (11.7)	14.8 (10.7)	0.926
CRP (mg/L)	9.9 (15.5)	6.4 (7.7)	0.273
Sputum inflammatory markers			
IL8 (pg/ml)	1246 (173)	1577 (158)	0.509
Myeloperoxidase (pg/ml)	243 (116)	150 (66)	0.707
Neutrophil elastase (pg/ml)	665 (183)	1468 (300)	0.628
Cholesterol levels			
Cholesterol (mmol/L)	5.1 (1.1)	5 (0.9)	0.6
Sputum microbiology			
Haemophilus influenzae	8 (27%)	6 (20%)	-
Streptococcus pneumoniae	4 (13%)	1 (3%)	-
Staphylococcus aureus	3 (10%)	2 (7%)	-
Other enteric gram negative organisms	3 (10%)	2 (7%)	-
Pseudomonas aeruginosa	2 (6%)	-	-
Moraxella catarrhalis	-	1 (3%)	-

Mixed normal flora	13 (43%)	17 (57%)	-
No sputum produced	-	1 (3%)	-
Pre treatment exercise capacity			
ISWT (m)	367 (158)	369 (128.8)	0.8
Other pre treatment medications			
Inhaled corticosteroids	22 (73%)	18 (60%)	0.07
Oral steroids	0%	2 (7%)	0.5
Long term antibiotic for chest (penicillin)	1 (3%)	1 (3%)	1
% DM requiring insulin	0%	0%	-
Leicester Cough Questionnaire			
Units	13.1 (4.0)	15.1 (4.4)	-

Table 2. Baseline characteristics of study population. P value indicates the differences between the groups at baseline; BMI = body mass index; CRP= C-reactive protein; FEV_1= Forced expiratory volume in 1 second; FVC= Forced vital capacity; ICS=inhaled corticosteroid; IgG_2= Immunoglobulin G_2; IHD=ischaemic heart disease; ISWT= incremental shuttle walk test.

* About a third of the patients were asthmatics in both groups- subanalysis of this group not done in this thesis.

3.3. AETIOLOGY

Most common etiology in both groups was idiopathic with post infectious and autoimmune cause being the next most common aetiologies. The aetiologies are summarized in table 3 and figure 1.

Aetiology	Group receiving atorvastatin	Group receiving placebo	P value
Idiopathic	21 (70%)	21 (70%)	1.00
Post infectious	4 (13%)	4 (13%)	1.00
Auto immune disease	4 (13%)	4 (13%)	1.00
Inflammatory bowel disease	1 (3%)	0	1.00
IgG_2 deficiency	0	1 (3%)	1.00

Table 3. Etiology of bronchiectasis in study patients

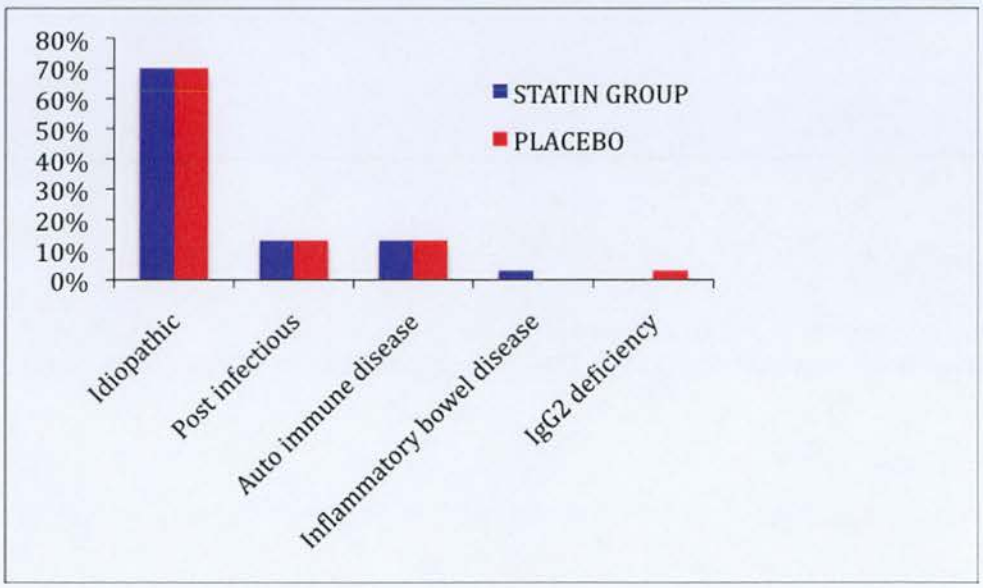


Figure 1. Aetiology of bronchiectasis in study patiemts.

3.4. PRIMARY OUTCOME

The change from baseline to 6 months in LCQ score differed between groups, with a mean change of 1.5 units in patients allocated atorvastatin versus –0.7 units in those assigned placebo (mean diff erence 2.2, 95% CI 0.5–3.9; p=0.01). 12 (40%) of 30 patients in the atorvastatin group improved by 1.3 units or more on the LCQ compared with five (17%) of 30 in theplacebo group (diff erence 23%, 95% CI 1–45; p=0.04) (Figure 2&3).

At 3 months

There was evidence of a difference in baseline to 3-month change in LCQ between the treatment groups, with a significant improvement in the statin treated group, with a mean difference 3.3, 95% CI for difference (0.9, 5.6) p=0.006.

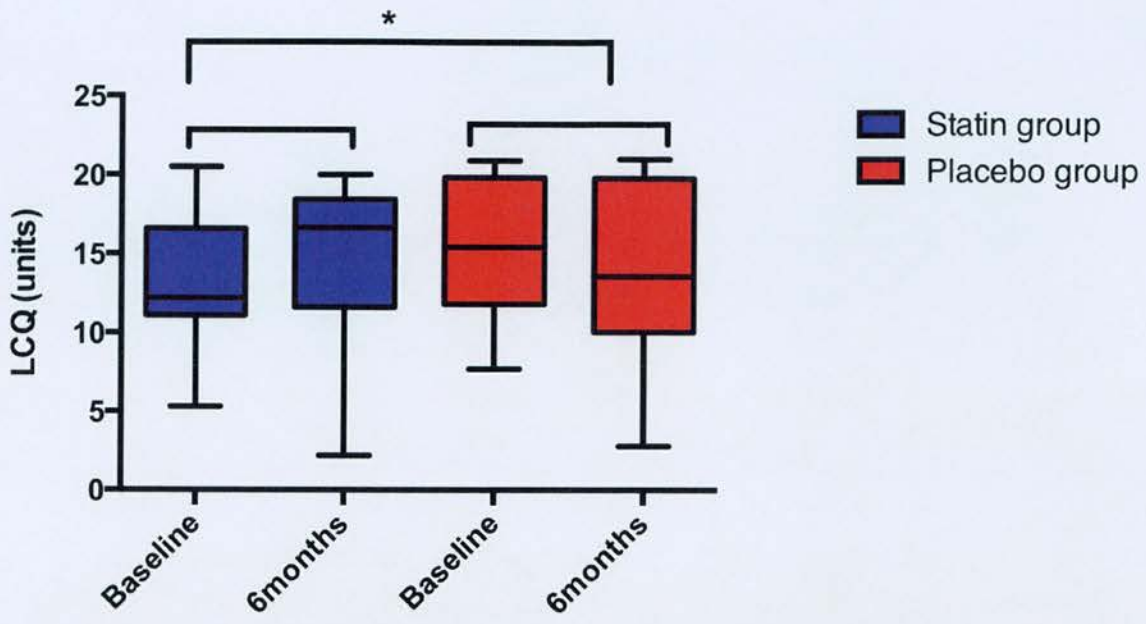
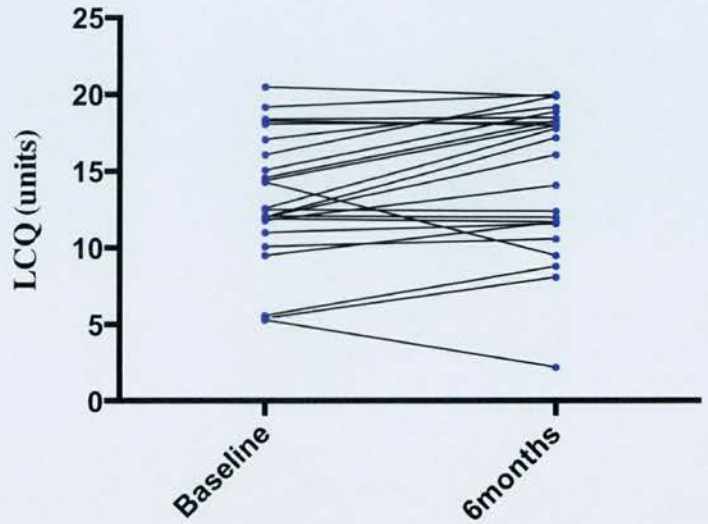
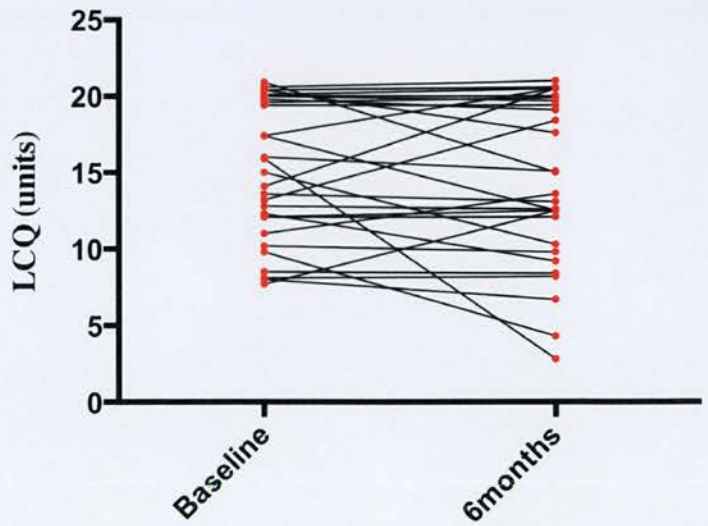


Figure 2: Reduction of cough in the statin group, as measured by LCQ; *p=0.01 for comparison of reduction in cough between both groups, at the end of 6months.

Figure 3. % of patients that had ≥ 1.3 Units improvement in the LCQ at the end of 6months treatment.



(a) STATIN GROUP



(b) PLACEBO GROUP

Figure 4. (a) Representative changes in LCQ in statin treated and (b) patients on placebo.

3.5. SECONDARY OUTCOMES

3.5.1. Sputum differential count

In the statin group, there was a decrease in the total number of sputum neutrophils and increase in number of apoptotic neutrophils/ 400 cells counted for each sample, with a median (IQR) difference of 5.5 (0.0, 15.0) in the number of apoptotic neutrophils/ 400 cells counted; p value 0.04 and -32.5 difference in the total number of neutrophils/ 400 cells; p=0.09; figure 5. At the end of 6-months of statin treatment the % change of viable neutrophils in the sputum was -19.5% and apoptotic neutrophils was 15.14%, compared to baseline, p=0.0001. In the placebo group, at 6-months % change of viable neutrophils was -1.4% and apoptotic neutrophils 1.4% compared to baseline, p=0.7; figure 5.

There was no significant difference in the number of eosinophils, basophils or monocytes, between active and placebo groups.

	Median (interquartile range) change [6-month to baseline]	
Outcome	**Atorvastatin N=24**	**Placebo N=29**
Sputum differential count **(per 400 cells counted)**		
Apoptotic neutrophil	5.5 (0.0, 15.0)	0.5 (0.0, 3.0)
Apoptotic neutrophil (% difference of apoptotic neutrophils)	15.14% (3%)	-1.4% (0.8%)
Neutrophil	-32.5 (-88.5, 1.5)	-0.5 (-25, 5)
Neutrophil (% difference of viable neutrophils)	-19.5% (5%)	1.4% (0.6%)
Basophil	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
Eosinophil	0.0 (0.0, 0.0)	0.0 (-0.5, 1.0)
Monocytes	-1 (-2.5, 1)	0.0 (0.0, 2)

Table 3. Median change (IQR) of sputum differential count, from baseline to end of treatment at 6months.

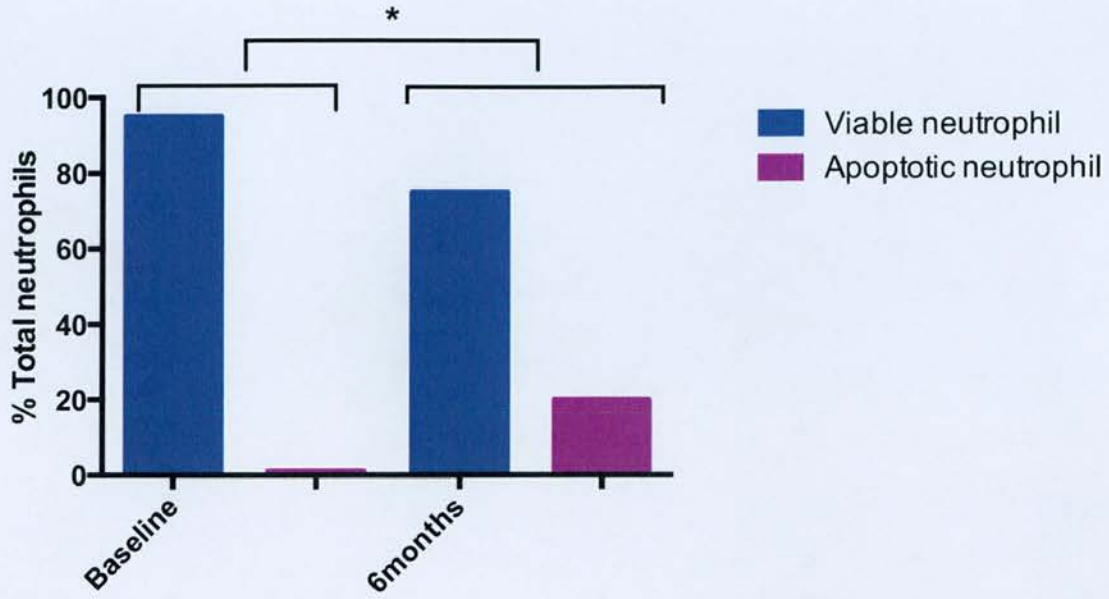


Fig 5a. % of viable and apoptotic neutrophils at baseline and end of 6months in the statin group; *p=0.0001.

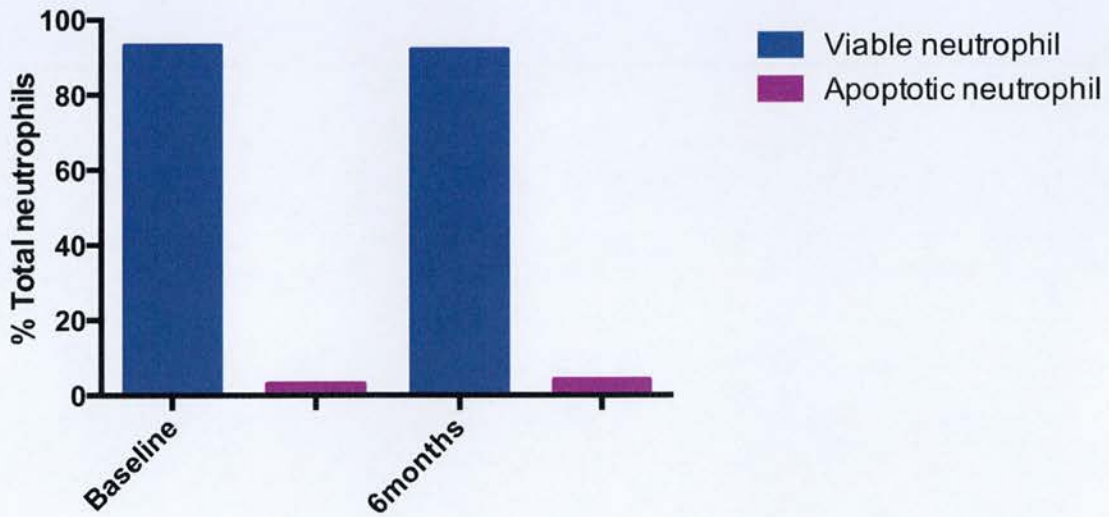


Figure 5b. % of viable and apoptotic neutrophils at baseline and end of 6months in the placebo group.

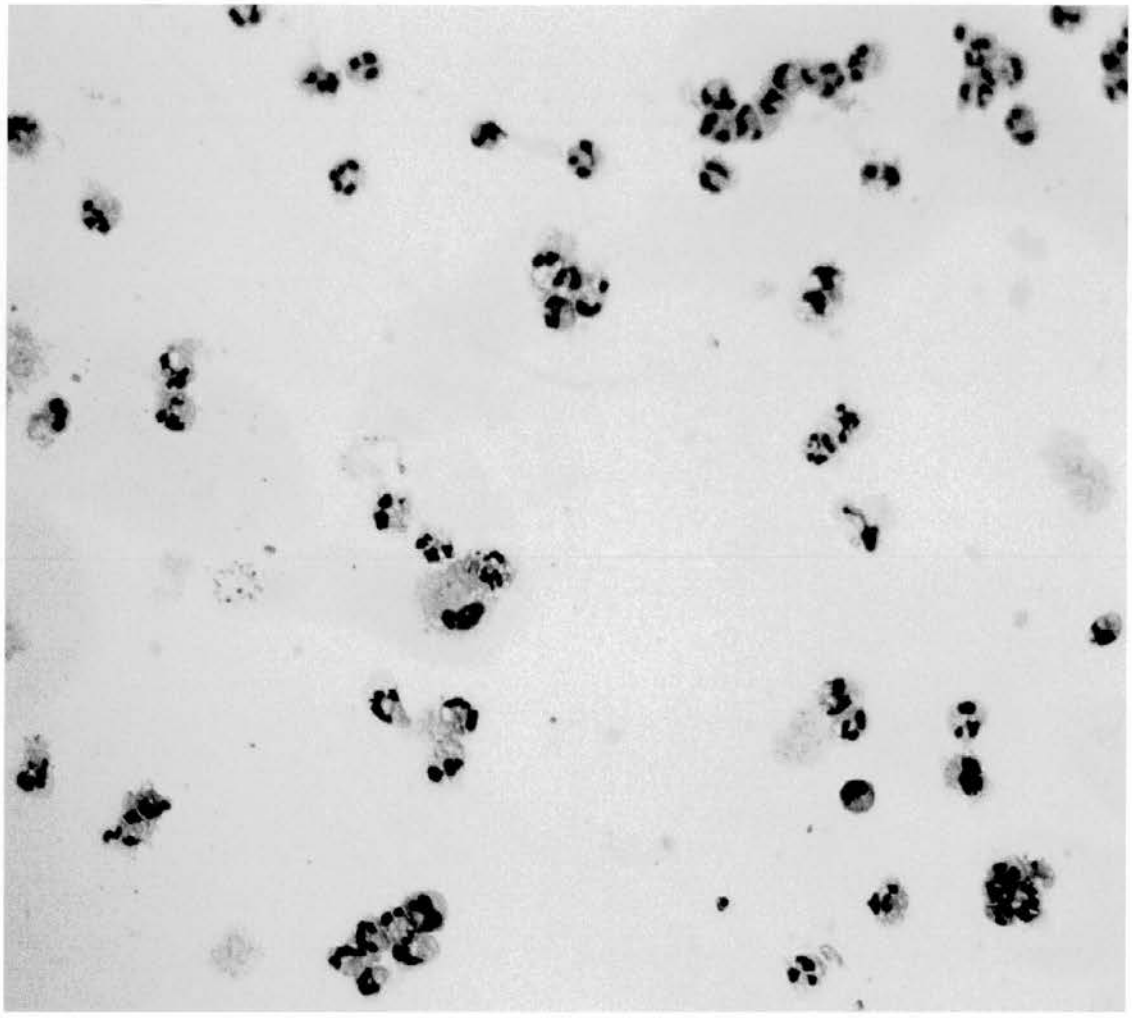


Figure. 6. Cytospin (x400 magnification) at baseline from patient on statin therapy.

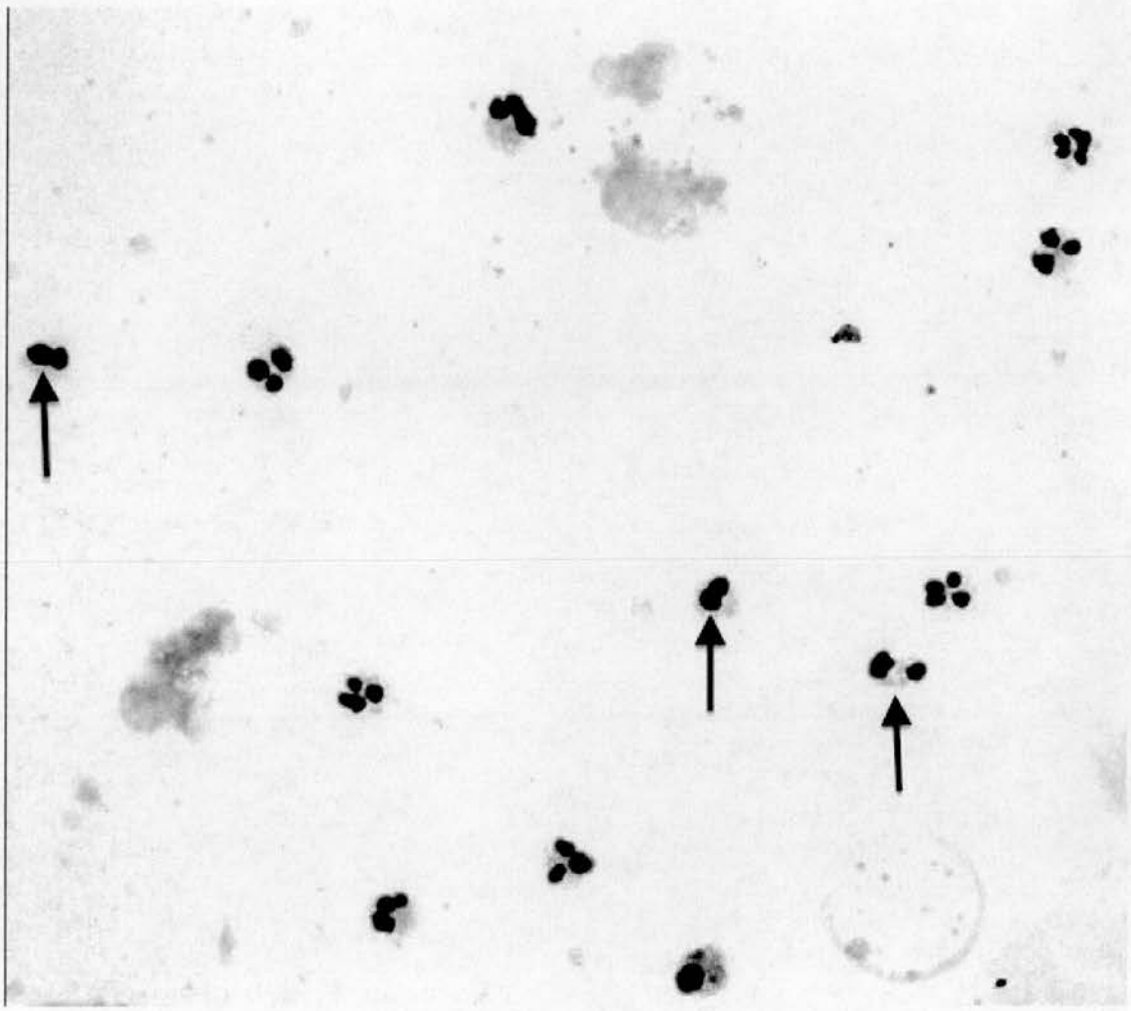


Figure 7. Cytsospin (x400 magnification) from same patient at the end of treatment with statins with lower number of viable neutrophils and an increase in the number of apoptotic neutrophils. Arrows indicate apoptotic neutrophils.

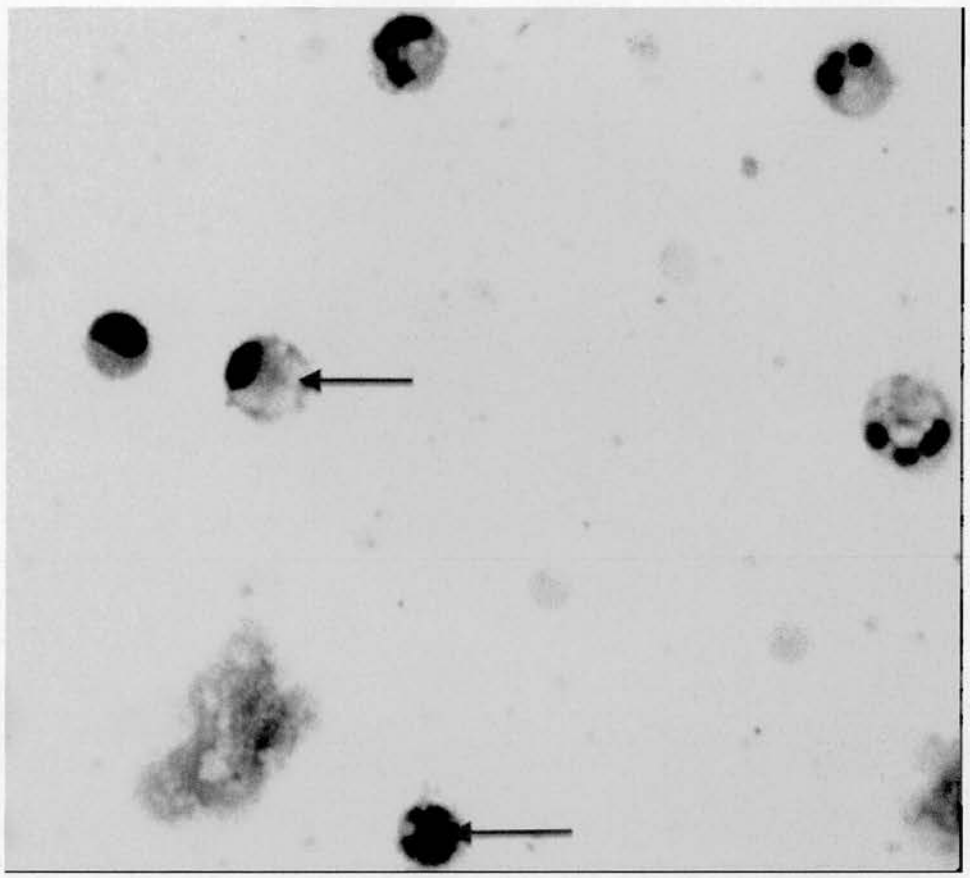


Figure 8. Increased levels of sputum neutrophil apoptosis following atorvastatin. Figure shows neutrophils (x1000) from sputum (per 400 cells counted) in a patient on 6-months statin therapy. Apoptotic neutrophils indicated by arrows.

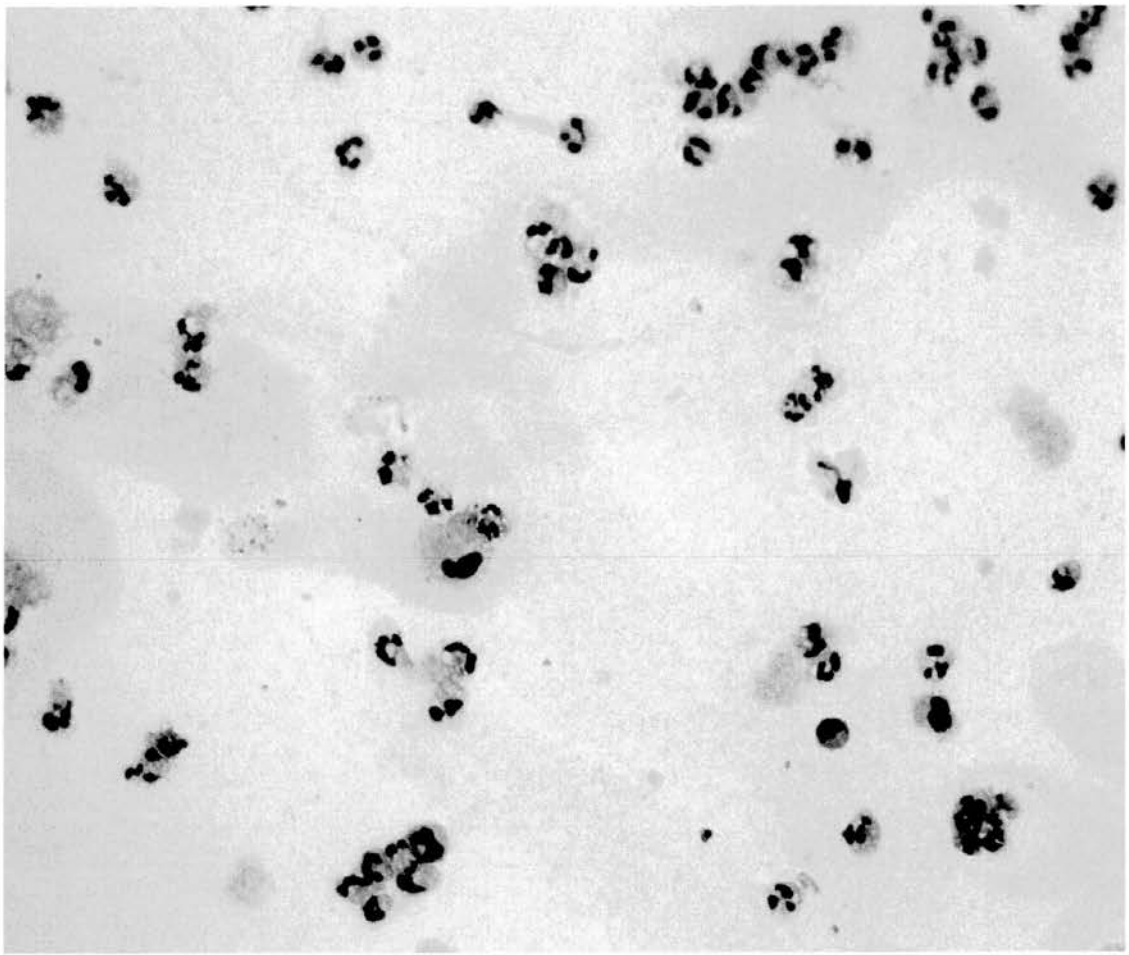


Figure 9. Cytospin (x400 magnification) at baseline from patient on placebo.

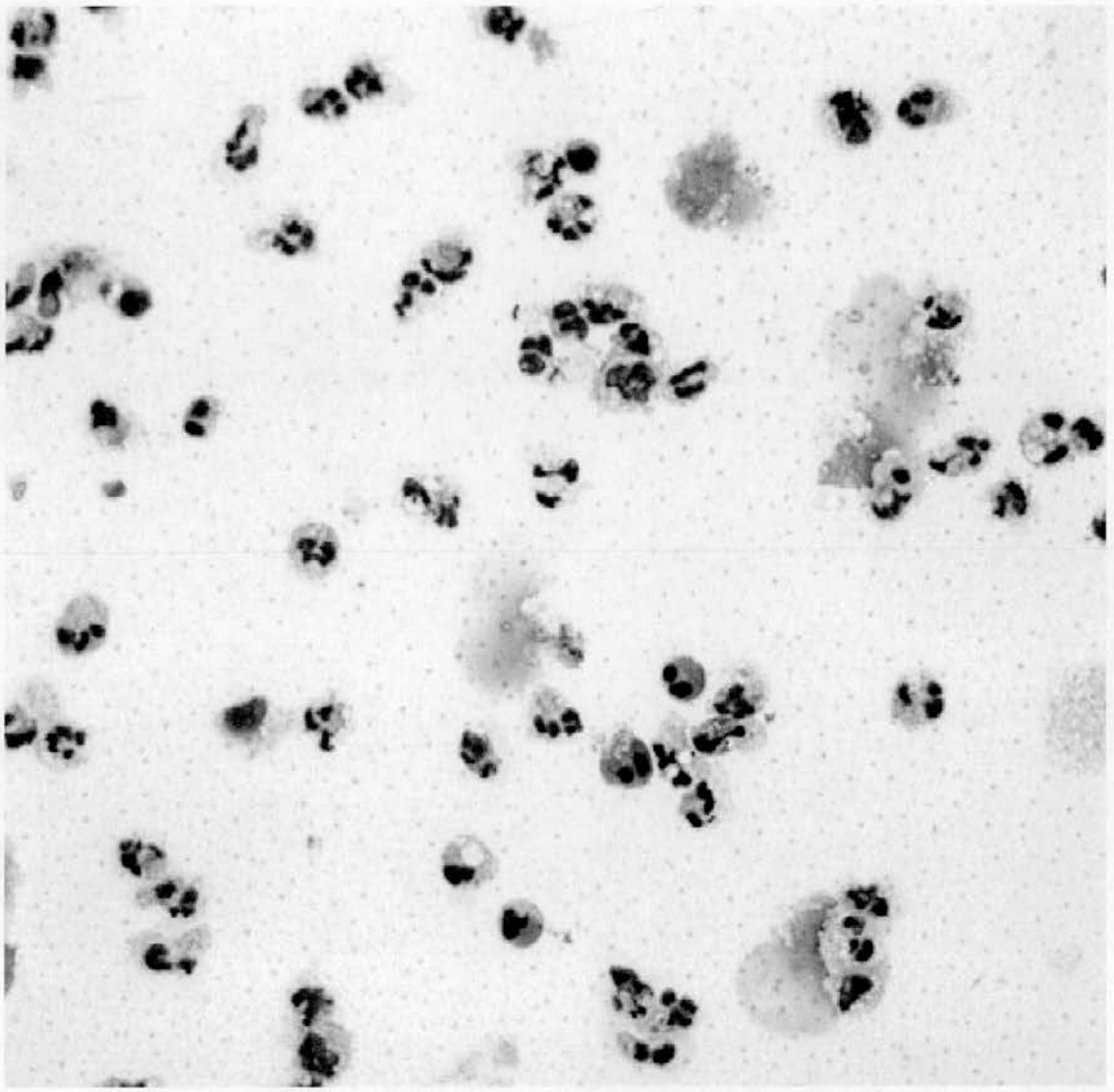


Figure 10. Cytsospin (x400 magnification) from same patient at the end of treatment with placebo. No evidence of apoptotic neutrophils or reduction in total number of viable neutrophils.

3.5.2. Sputum inflammatory markers

There is no significant difference in the baseline to 6month change in IL-8, MPO or free NE between those treated with statin compared to placebo, p value= 0.6, 0.6 and 0.9 respectively (table 4).

	Median (interquartile range) change [6-month to baseline]			
Outcome	**Atorvastatin N=24**	**Placebo N=29**	**95% CI of change**	**P value**
Interleukin-8 (pg/ml)	254 (-968, 1075)	260 (-312, 752)	-167.3 (-815.6, 481.1)	0.607
Myeloperoxidase (pg/ml)	0 (-8.5, 32.5)	0 (-21, 56)	117.5 (-388.8, 623.9)	0.638
Neutrophil elastase (pg/ml)	0 (-638, 194)	0 (-1034, 360)	10.6 (-1689.1, 1728.3)	0.982

Table 4. Median change (IQR) of sputum inflammatory markers, from baseline to end of treatment at 6months.

3.5.3. Systemic Inflammation

There was a significant reduction in IL-8 in the atorvastatin group at 6 months; p=0.03 (figure 11, table 5), compared to baseline. However, there was no affect on the IL-1β, IL-6, IL-10, IL12p70 or TNF-alpha levels.

There was no statistically significant difference in the baseline to 6-month change in leucocyte count, total neutrophil count or ESR in the atorvastatin treated group compared to the placebo group. However, CRP levels decreased in the atorvastatin group (but just failed to reach statistical significance); p=0.06 (table 5).

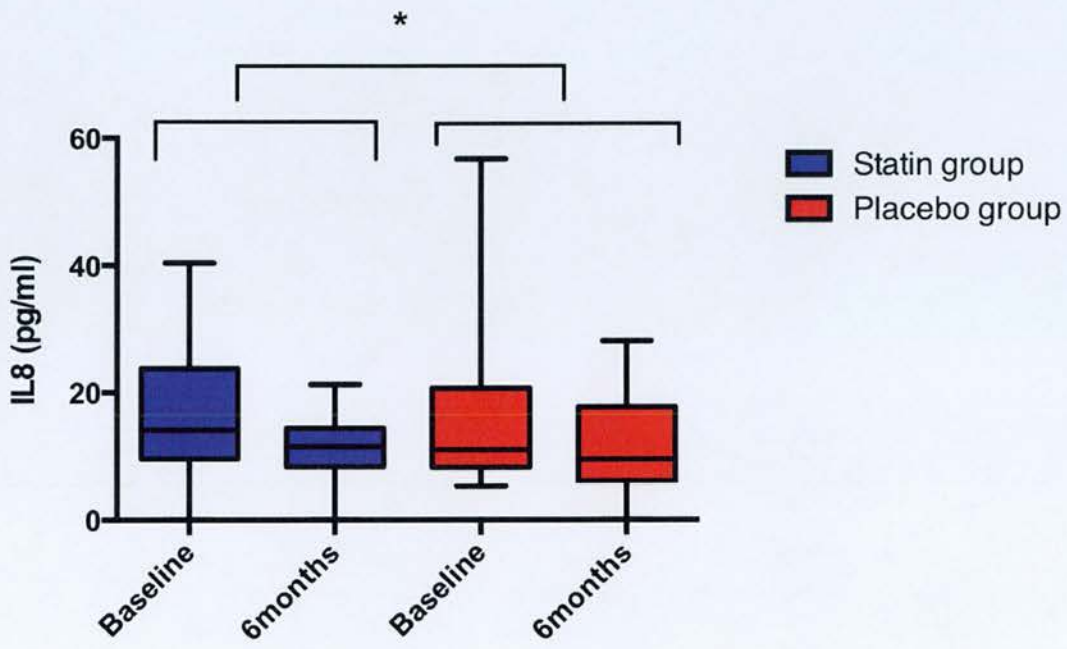


Figure 11. Serum IL8 measured at baseline and at 6 months; *p=0.03.

	Median (interquartile range) change [6-month to baseline]		
Outcome	**Atorvastatin N=24**	**Placebo N=29**	**P value**
White blood cells ($X10^9$/L)	-0.4 (-0.7, 0.5)	-0.1 (-0.8, 0.9)	0.863
Neutrophils ($X10^9$/L)	-0.0 (-0.4, 0.4)	-0.1 (-0.5, 0.9)	0.914
Lymphocytes ($X10^9$/L)	-0.1 (-0.3, 0.1)	-0.0 (-0.2, 0.1)	0.805
Monocytes ($X10^9$/L)	-0.0 (-0.1, 0.1)	-0.1 (-0.1, 0.0)	0.174
Eosinophils ($X10^9$/L)	-0.1 (-0.1, 0.0)	-0.0 (-0.1, 0.1)	0.310
CRP (mg/L)	-1.0 (-6.0, 0.0)	0 (-3.0, 1.0)	0.058
ESR (mm/hr)	-1.0 (-8.0, 1.0)	1.0 (-4.0, 6.0)	0.569
IL-8 (pg/ml)	-1.9 (-0.4, 18.8)	0.3 (-4.2, 7)	0.03

Table 5. Median change (IQR) of sputum inflammatory markers, from baseline to end of treatment at 6months.

* unpaired t test done for IL8.

3.5.4. Spirometry

There was no difference in the baseline to 6-month change by treatment for FEV_1, FVC or FEV_1:FVC ratio (table 6).

3.5.5. Exercise Capacity

There was a trend towards improvement in the baseline to 6-month change by treatment, p=0.07 (table 6).

	Median (interquartile range) change [6-month to baseline]		
Outcome	**Atorvastatin N=24**	**Placebo N=29**	**P value**
Spirometry			
FEV_1 (L)	-0.01 (-0.13, 0.13)	0.06 (-0.06, 0.17)	0.894
FVC (L)	-0.08 (-0.3, 0.13)	-0.07 (-0.34, 0.2)	0.495
FEV_1: FVC	0.01 (-0.02, 0.07)	0 (-0.04, 0.07)	0.799
Exercise capacity (Incremental shuttle walk test)			
Distance walked (m)	35 (-10, 95)	0 (-20,40)	0.07

Table 6. Median change (IQR) of spirometry and incremental shuttle walk test, from baseline to end of treatment at 6months.

3.5.6. Exacerbation Frequency

8/24 (33%) of patients on statin had 2 or more exacerbations compared to 16/29 (55%) in the placebo group, with a relative risk ratio of 0.6 (95% CI 0.3-1.2).

5/24 (21%) of patients on statin had 3 or more exacerbations compared to 10/29 (34%) in the placebo group, with a relative risk ratio of 0.6 (0.2-1.5) (table 7).

Exacerbations	Statin group N (%)	Placebo N (%)
0	6 (25%)	9 (31%)
1	10 (42%)	4 (14%)
2	3 (13%)	6 (21%)
≥3	5 (21%)	10 (34%)

Table 7. Exacerbations in both groups while on treatment.

3.5.7. Comparison of exacerbations while on therapy to 6months before start of trial

At baseline median number of exacerbations in the statin group were 3 (1-5.5) and in the placebo were 2 (0-3); pvalue 0.04.

While on treatment for 6months, number of exacerbations in the statin group were 1 (0.5-2) and in the placebo were 2 (1-4). Comparison of exacerbations were done in both groups and is demonstrated graphically in figure 13. Wilcoxon signed rank test was done on the difference in exacerbation frequencies recorded, in either group. In the statin treated group, there was a significant reduction in exacerbation frequency at the end of statin therapy compared to 6months before, p=0.001.

There was no significant change in the placebo group at the end of treatment compared to 6months before, p=0.67. However, given the difference in baseline exacerbations, the significance of these results should be carefully interpreted.

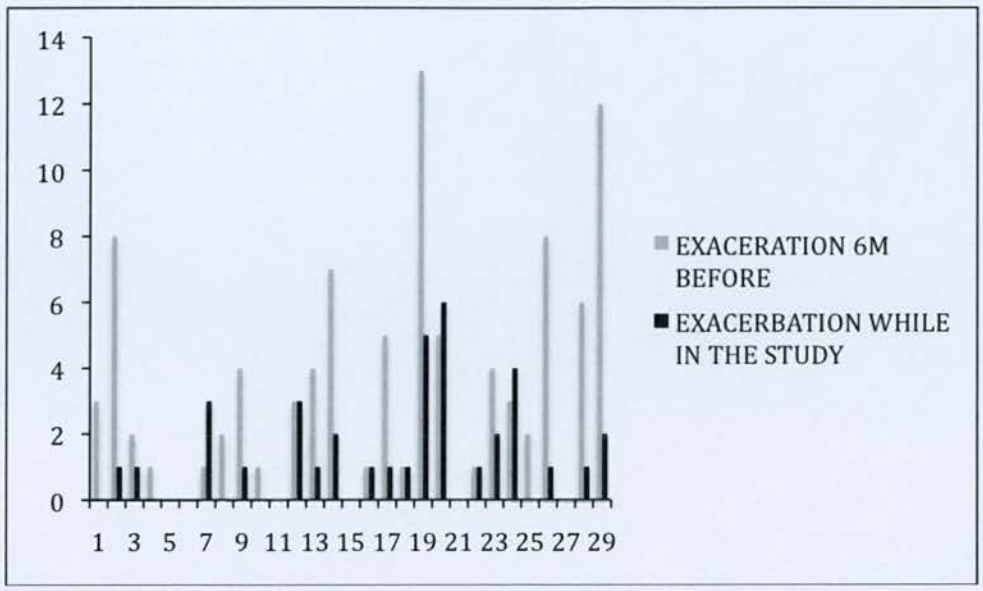


Figure 12. Exacerbations 6months before and during the 6months on the study- statin group (days along y- axis and individual patients along x-axis).

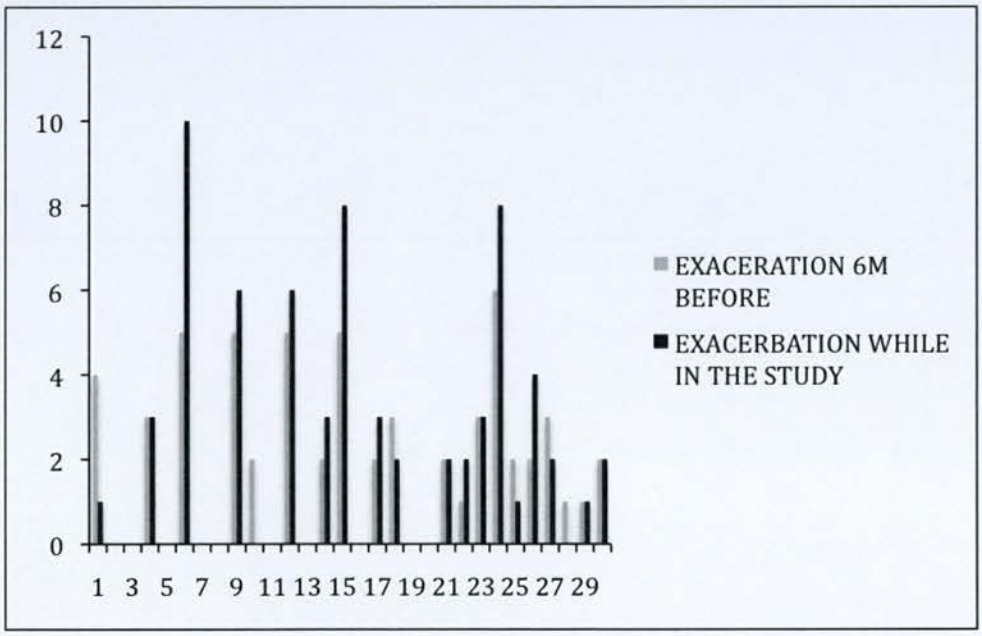


Figure 13. Exacerbations 6months before and during the 6months on the study- placebo group (days along y- axis and individual patients along x-axis).

3.5.8. Sputum Microbiology

Haemophilus influenzae was the most common colonizing organism in both groups at baseline. At the end of treatment, 63% were colonized with microorganisms in the statin group (57% at baseline), compared to 41% in the placebo group (43% at baseline); p=0.1 for end of treatment proportion. There was no difference in bacterial load when comparing the baseline to 6-month change on the basis of treatment. Mean change in bacterial load was $-2.7x10^7$ cfu/ml after 6-months of statin therapy p=0.1. In the placebo group, mean change in bacterial load at the end of treatment $1.9x10^7$cfu/ml; p=0.1, table 8.

3.5.9. Quality of life- St. George's Respiratory Questionnaire

There was no evidence of improved quality of life in the atorvastatin treatment group, difference in proportion -13%, 95% CI for difference (-29%, 3%) p=0.2 (table 8).

	Mean change [6-month to baseline] SD		
Outcome	**Atorvastatin N=24**	**Placebo N=29**	**P value**
Sputum microbiology			
Colonized at baseline	57%	43%	0.1
Colonized at 6months	63%	41%	0.2
Bacterial load at baseline (cfu/ml)	$3.83x10^7$	$1.11x10^7$	0.1
Bacterial load at 6months (cfu/ml)	$1.17x10^7$	$3x10^7$	0.1
St. George's Respiratory Questionnaire			

No (<4unit improvement)	**23 (96%)**	**24 (83%)**	
Yes (≥ 4 unit improvement)	**1 (4%)**	**5 (17%)**	**0.2**

Table 8. Median change (IQR) of sputum microbiology and quality of life questionnaire, from baseline to end of treatment at 6months.

3.5.10. Routine blood tests

There was no statistically significant difference in the baseline to 6-month change in urea, creatinine, alanine aminotransferase (ALT), or creatinine kinase levels in those treated with statin compared to placebo. However, there was a difference in the baseline to 6-month change in cholesterol in the statin group exhibiting a greater drop, difference in means -1.40, 95% CI (-1.77, -1.02); p<0.0001 (table 9).

	Mean change [6-month to baseline] SD		
Outcome	**Atorvastatin N=24**	**Placebo N=29**	**P value**
ALT (U/L)	2.0 (-5.5, 5.5)	-1.0 (-5.0, 4.0)	0.475
CK (U/L)	-5.5 (-49.0, 21.5)	-20.0 (-61, -3.0)	0.131
Cholesterol (mmol/L)	-1.3 (-2.1, -0.6)	0 (-0.2, 0.4)	<0.0001
Urea (mmol/L)	0.3 (-0.9, 1.1)	0.2 (-0.8, 0.9)	0.863
Creatinine (μmol/L)	-1.0 (-4.0, 3.0)	-1.0 (-4.0, 7.0)	0.597

Table 9. Mean change (SD) with 95% CI of routine blood tests, from baseline to end of treatment at 6months.

3.5.11. Comparison of variables where compliant

Using the baseline to 6-month change in cholesterol levels as an indicator of compliance, a stratified analysis was conducted.

In the placebo group, 27/29 did not have a reduction of 1mmol/L point or more in cholesterol and in the atorvastatin group, 15/24 had a reduction of 1mmol/L or more. Within these patients, there is evidence of a difference in baseline to 6-month change in LCQ between the treatment groups, difference 2.2, 95% CI for difference (0.4, 3.9) p=0.016. This sub analysis shows an improvement in LCQin the statin treated group, compared to the placebo group.

3.6. Adverse Events and Safety

Ten of 30 (33%) patients had an adverse event in the statin group compared to three of 30 (10%) in the placebo, difference in proportion 23% (95% CI for difference 3%, 43%), p=0.02. There were however no serious adverse events.

2/30 (7%) patients in the statin group developed leg pain in the first week of starting the treatment, but this subsided in the second week for both patients. 1/30 (3%) in the statin group and 2/30 (7%) in the placebo group had raised creatinine kinase (CK) while on treatment (<three times upper limit of normal). This was detected at 3 months after starting treatment. These patients had repeat CK levels measured after 1 week and there was either a reduction in the levels or the levels had normalized. No patients had to withdraw from the study due to high CK levels. In the statin group, 1/30 (3%) developed headache, while on treatment and had to withdraw from the study due to it. Diarrhoea was reported in 1/30 (3%) patient within 1 week of starting statin treatment and had to discontinue due to persisting diarrhea. 2/30 (7%) patients developed both diarrhoea and headache whilst on statin treatment and both had to withdraw from the study, due to persisting symptoms. 1/30 (3%) of the patients in both groups developed abdominal discomfort one week after starting treatment, but this improved spontaneously and did not need to stop the trial therapy. At 3 months, 1/30 (3%) patient in the statin group had an ALT of 325 U/L and, as this was >5times normal, the patient was withdrawn from the study. Checking ALT, three days after stopping treatment with statin, showed that it had normalized. 1/30 (3%) patient in the statin group developed haematuria during the first week of starting the treatment. However, he was known to have renal calculi, surgeons reviewed him to confirm this, and he underwent lithotripsy for the renal calculi and continued the study treatment. All patients had normal renal function throughout the study, when measured at baseline and 6-months; table 2.

Adverse events of the study are summarized in table 10.

Adverse events	Statin group N=30	Placebo group N=30
Leg pain	2 (7%)	0
Raised CK	1 (3%)	2 (7%)
Headache	3 (10%)	0
Diarrhea	3 (10%)	0
Abdominal discomfort	1 (3%)	1 (3%)
Deranged LFTs	1 (3%)	0
Haematutria	1(3%)	0

Table 10. Adverse events in both groups.

CHAPTER 4

MECHANISTIC STUDIES

4.1. KEY STUDY FINDINGS

In summary, atorvastatin 80mg orally, daily for 6months in patients with moderately severe bronchiectasis, led to reduced cough (as measured by the Leicester Cough Questionnaire); increased number of apoptotic sputum neutrophils at the end of treatment and a significant reduction in serum IL8. Although the mechanism for reduction in cough is not clear, we hypothesize that statin enhance apoptosis of sputum neutrophils, thereby promoting resolution of inflammation, which is beneficial in bronchiectasis.

There is limited data available in the literature on apoptosis of airway neutrophils in bronchiectasis. Two important studies have discussed airway neutrophil apoptosis in bronchiectasis. Vandivier *et al* concluded that sputum of bronchiectatic patients have more apoptotic neutrophils in comparison to sputum obtained from patients with chronic bronchitis. Although they attribute that presence of increased apoptotic cells could be secondary to several key mechanism, they showed through *in vitro* studies that failure of clearance of apoptotic neutrophils by macrophages was perhaps one of the key contributors to persistence of apoptotic cells. They were able to demonstrate that free neutrophil elastase (NE), which is present in abundance in bronchiectatic airways, cleaves the phosphotidylserine receptor on macrophages, rendering poor efferocytosis. Hence persistence of inflammatory cells in the airways promotes further inflammation leading to failure of resolution.

In comparison, a study by Watt *et al* showed that in bronchiectasis, there were low levels of apoptotic neutrophils in induced sputum, as compared to the findings by Vandivier. They found no significant differences in the levels of apoptotic or necrotic neutrophils at the beginning and end of an exacerbation treated with antibiotics. There was however a reduction in the total number of sputum neutrophils and serum levels of IL-8, TNF-α, NE and CRP were reduced.

In summary, few studies have investigated neutrophil apoptosis in bronchiectasis. Of the two key studies described to date, the opinion remains divided. In view of our study findings of increase in sputum neutrophil apoptosis after 6 months of statin treatment and the paucity of data on neutrophil apoptosis in bronchiectasis, we investigated the role of statins on neutrophil apoptosis and possible mechanisms by which statins induce apoptosis.

4.2. APOPTOSIS AND EFFEROCYTOSIS

It is known that in bronchiectasis, there is excessive neutrophilic airways inflammation. However, despite this, over two thirds of patients are chronically colonized with microorganisms. This apparent paradox in bronchiectasis needs to be explored further. Granulocyte apoptosis and their removal by phagocytes, particularly macrophages, are essential for resolution of inflammation (Greening and Simon 2011). The neutrophil, which although short-lived in the circulation, is granted an extended life span by survival signals at inflammatory sites (Lee et al 1993). Apoptosis renders these potentially histotoxic cells functionally redundant and ensures their recognition and clearance by macrophages (Savill et al 1989). It is known that phagocytosis of apoptotic cells (efferocytosis) is integrally involved with the regulation of the inflammatory response and maintenance of lung homeostasis by 1) removing dead cells before the onset of necrosis (Henson et al 2001), by 2) inducing release of anti-inflammatory mediators (Henson et al 2001) and antiproteases (Odaka et al 2003), and by 3) increasing production of growth factors (Morimoto et al 2001, Golpon et al 2004). Hence impaired efferocytosis is involved in the pathogenesis of a variety of chronic inflammatory lung diseases such as cystic fibrosis, bronchiectasis, asthma and COPD (Vandivier et al 2002, Hodge et al 2003, Bratton and Henson 2005). It is essential for activated neutrophils to be turned off, for resolution of inflammation to take place. Neutrophils that are over-activated or undergo necrosis release their toxic granule contents, causing unwanted tissue damage. By inducing neutrophil apoptosis together with efficient macrophage removal, it is possible to promote the resolution of inflammation (figure 1) (Ren et al 2008).

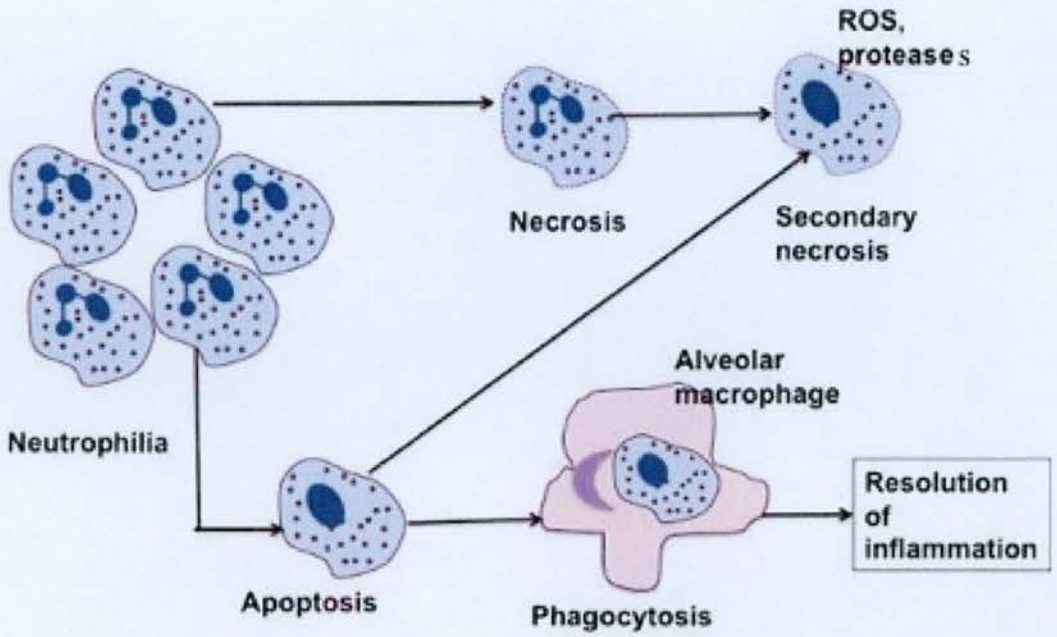


Figure 1. Diagram showing resolution and failure of resolution of neutrophil mediated inflammatory process. Neutrophilia is resolved by apoptosis and clearance by macrophages. Failure of resolution after apoptosis, where neutrophils are in excess or are not cleared leads to secondary necrosis. ROS= Reactive oxygen species.

4.3. ATORVASTATIN

Mechanism of action: Inhibition of HMG CoA reductase

Statins target hepatocytes and inhibit HMG-CoA reductase, the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor. Statins do more than just compete with the normal substrate in the enzymes active site. They alter the conformation of the enzyme when they bind to its active site. This prevents HMG-CoA reductase from attaining a functional structure. The change in conformation at the active site makes these drugs very effective and specific. Binding of statins to HMG- CoA reductase is reversible, and their affinity for the enzyme is in the nanomolar range, as compared to the natural substrate, which has micromolar affinity (Corsini et al 1999). The inhibition of HMG-CoA reductase determines the reduction of intracellular cholesterol, inducing the activation of a protease that slices the sterol regulatory element binding proteins (SREBPs) from the endoplasmic reticulum. SREBPs are translocated at the level of the nucleus, where they increase the gene expression for LDL receptor. The reduction of cholesterol in hepatocytes leads to the increase of hepatic LDL receptors, that determines the reduction of circulating LDL and of its precursors (intermediate density-IDL and very low density-VLDL lipoproteins) (Sehayak et al 1994). All statins reduce LDL cholesterol non-linearly, dose-dependent, and after administration of a single daily dose (Blum 1994). Efficacy on triglyceride reduction parallels LDL cholesterol reduction (Stein et al 1994). By inhibiting mevalonic acid synthesis, statins also prevent the synthesis of other important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Evans and Rees 2002). These isoprenoids are necessary for the post translational modification and subsequent trafficking of intracellular signalling molecules.

Inhibition of HMG-CoA reductase, the regulatory enzyme of the pathway, results in disturbances in practically all vital cellular processes, such as protein glycosylation and prenylation, cell signaling, functioning of the respiratory chain and integrity of cellular membranes (Buhaescu and Izzedine 2007). The impairment of these processes may contribute to the pleiotropic side effects of statins (Evans and Rees 2002, Onfrei et al 2008, Zhou and Liao 2009). The pleiotropic effects of statins have

now been studied extensively. Of significance to us are the anti inflammatory effects of statins (figure 2).

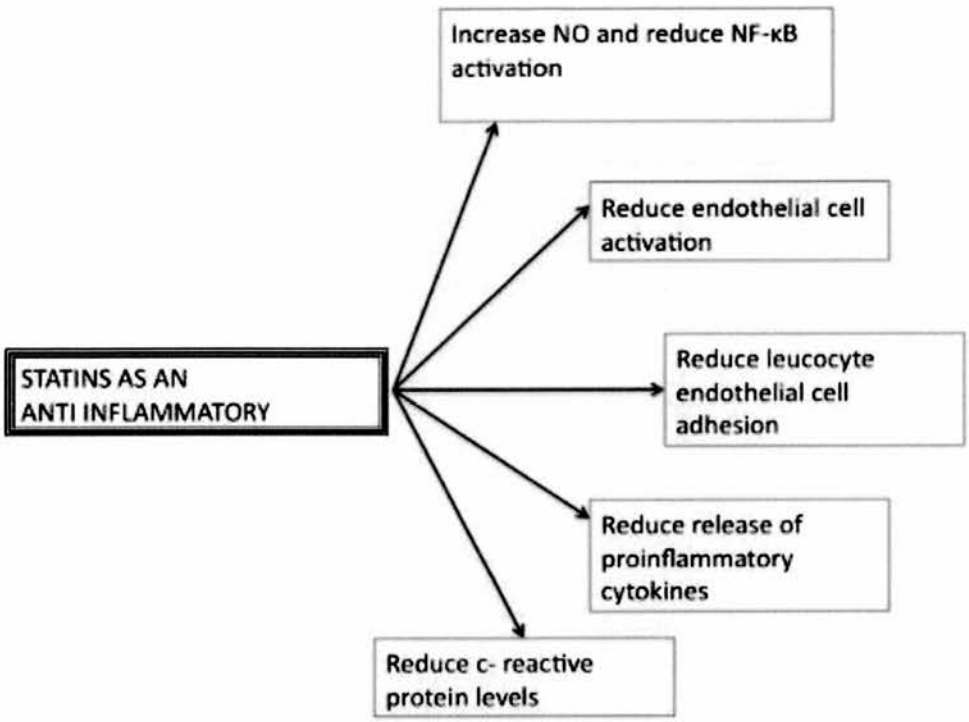


Figure 2. Anti inflammatory effects of statins. NFκB= nuclear factor κB cells: NO= nitric oxide.

4.4. STATINS AND LUNG INFLAMMATION

Several studies have documented the beneficial effects of statins *in vitro* and *in vivo* in experimental animal models of pulmonary inflammation. Shyamsundar *et al* first reported the effects of statin *in vivo* on LPS induced lung inflammation in humans. Briefly, in this RCT, healthy volunteers were given simvastatin for 4 days following which they were challenged with inhaled LPS. Bronchoalveolar lavage (BAL) was performed at 6 hours after challenge and serum was collected after 24 hours. Pre treatment with simvastatin reduced LPS induced BALF neutrophilia, MPO, TNF-α, matrix metalloproteinases 7, 8 and 9 as well as plasma CRP and had reduced upregulation of nuclear factor (NF) κB, as compared to control subjects. In addition, pretreatment with simvastatin significantly increased the number of neutrophils in late apoptosis, as compared to placebo. This data was obtained from 15 subjects in the active group and 9 healthy controls.

This was the first study to report the increase in pulmonary neutrophil apoptosis *in vivo* in humans when pretreated with statin, following acute lung injury stimulation.

The neutrophil apoptosis findings from this study were similar to our study findings. After 6 months of treatment with Atorvastatin 80mg, there was an increase in the number of apoptotic sputum neutrophils as compared to those who were treated with 6months of placebo.

We hypothesize that statins enhance apoptosis by regulating calcium flux. Hence it is of relevance to discuss key mediators such as roscovitine, fMLF, formyl peptide receptor 1 and cyclosporin H, which are involved in calcium flux.

4.5. MEVALONIC ACID

Formula: $C_6H_{12}O_4$

Systematic name: (3*R*)-3,5-Dihydroxy-3-methylpentanoic acid

Molar mass: 148.16 g/mol

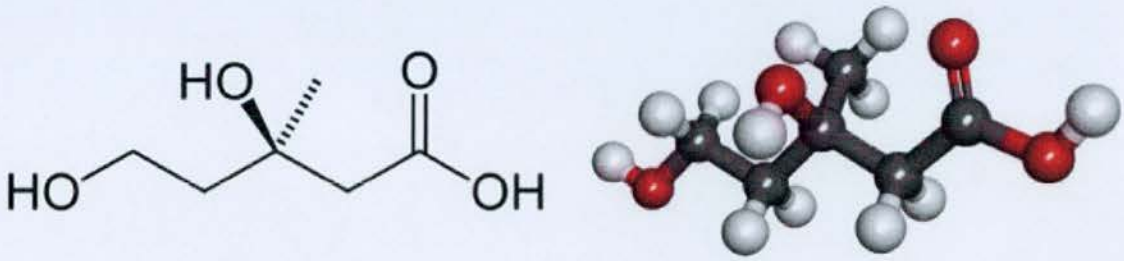


Figure 3. Mevalonic acid

Mevalonic acid (MVA) (derived from dihydroxymethylvalerolactone) is a key organic compound in the mevalonic acid pathway that leads to the synthesis of sterol isoprenoids, with the final product cholesterol, and nonsterol isoprenoids, such as dolichols, the side chain of ubiquinone, farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). Statins inhibit HMG CoA reductase which is the rate limiting step (see figure below). Hence blocking the production of mevalonic acid inhibits the production of the key final products of farnesyl diphosphate and cholesterol (figure 4). Replacement of mevalonic acid after inhibition by statins should thereby reverse the effects of statins.

Figure 4. Key steps in the mevalonic acid pathway.

4.6. ROSCOVITINE

Formula: $C_{19}H_{26}N_6O$

Systemic name: 2-(*R*)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine

Molar mass: 354.45 g/mol

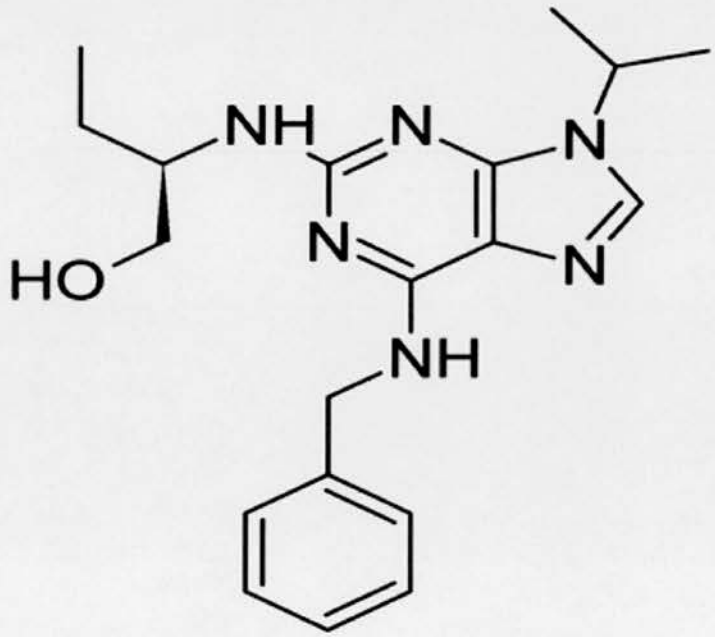


Figure 5. Roscovitine

Neutrophils are key to the immune system and are recruited rapidly to the site of inflammation. Neutrophils then release products of degranulation and activation to defend and destroy invading microorganisms but these byproducts are detrimental to the surrounding tissues (Nathan 2002). Activated neutrophils need to 'switch off' and undergo apoptosis for the resolution of inflammation. Once apoptosis has been engaged, the neutrophil secretory activity is shutdown; the cells remain intact and are phagocytosed by macrophages using recognition mechanisms (efferocytosis) that fail to elicit a pro-inflammatory response (Savill et al 1989, Whyte et al 1993). Impairment of either apoptosis or efferocytosis will lead to persistence of inflammation.

Neutrophil apoptosis is controlled by a complex network of signalling pathways that regulate both the turnover of key molecules, including the anti-apoptotic proteinmyeloid cell leukemia 1 (Mcl-1) and the pro-apoptotic Bcl-2 family member Bax, and activation of the caspase family of proteases. (Riley et al 2006).

Cell division of eukaryotic cells occurs in four phases (G1, S, G2, M) and in some circumstances, where the cell is terminally differentiated, the cell will rest in G0 phase. Neutrophils are terminally differentiated cells. The cyclin dependent kinases (CDK) are key regulators of the cell cycle, whereby different CDKs become activated during cell-cycle progression when complexed with their associated cyclin partners (Vermeulen et al 2003).

Rossi *et al* have been investigating the role of CDK inhibitors in neutrophil apoptosis, both *in vitro* and *in vivo* (Rossi et al 2006). They have shown that human neutrophils express functional CDKs and that different CDK inhibitors directly induce caspase-dependent neutrophil apoptosis and inhibit cell survival induced by several biologically important powerful anti- apoptotic agents. In addition, the CDK inhibitor R-roscovitine downregulates Mcl-1 expression induced by survival factors in neutrophils. Further studies have demonstrated *in vivo* that roscovitine markedly enhances resolution of inflammation in mouse models of carrageenan-induced acute pleurisy, bleomycin-induced lung inflammation and passively induced arthritis. The roscovitine enhanced resolution of established pleurisy is driven by a caspase-mediated pro-apoptotic effect (Rossi et al 2006).

Perhaps unsurprisingly, given the terminally differentiated state of these cells, the most significantly expressed CDKs (McGrath et al 2011, Leuenroth et al 2000) have no direct role in the cell cycle but are essential for transcription of a key subset of genes. Mcl-1 (pro survival protein) is down regulated to the level of genes and this is essential for the initiation of apoptosis. It has been further demonstrated that roscovitine manipulates the transcriptional machinery of the neutrophils to thereby promote apoptosis (Leitch et al 2012).
These findings suggest that CDK inhibitors may provide a therapeutic strategy to promote resolution of inflammatory diseases, through specific induction of inflammatory cell apoptosis.

4.7. N- FORMYL- METHYL- LEUCYL- PHENYLALANINE (fMLF)

Neutrophils are first responders in an organism's rapid assault on infectious pathogens. Through genetically conserved receptors, neutrophils recognize chemoattractants, lipid products, and the molecular patterns present on the surface of bacteria, viruses, and fungi (Medzhitoov and Janeway 2000). fMLF is recognized by neutrophils and is a potent neutrophil chemoattractant. fMLF, upon binding to its heterotrimeric G protein-coupled receptor, initiates signaling cascades that activate multiple pathways (Haribabu et al 2000). These pathways include the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI-3K) cascades, which are important for the development of the functional responses of neutrophils in inflammation (e.g., the respiratory burst, transmigration, and phagocytosis) (Mocsai et al 2000, Rane et al 1997, Della Bianca et al 1993, Coffer et al 1998).

4.8. FORMYL PEPTIDE RECEPTOR (FPR) 1

The FPR receptor family was extensively characterised throughout the 1980s as seven membrane spanning, G-protein coupled receptors (GPCR) via pertussis toxin sensitivity; specifically attributed to G-protein coupling (Lavigne et al 2002). The human family was cloned in 1990 (Boulay et al 1990) with three genes encoding FPR1, FPR2/ALX and FPR3 clustered on chromosome 19q13.3.

FPR1, the first chemotactic receptor to have its primary structure delineated by molecular cloning (Boulay et al 1990), has been extensively studied for its high affinity binding to fMLF. Following agonist ligation, FPR1 undergoes rapid phosphorylation in a concentration and time dependent manner (Ali et al., 1993) leading to conformation changes. (Migeotte et al 2006). Downstream of G-protein interaction there are a number of signalling pathways including calcium (Ca^{2+}) flux, phospholipase (PL) A, C and D, phosphoinositide-3-kinase (PI3K) and MAP kinase pathways (Selvatici et al 2006).

PLC is an essential upstream mediator of both PI3K and mitogen- activated protein kinase pathways via protein kinase C (PKC). PLA is well characterised for mediating

PLC activation central to the biosynthesis of eicosanoids. In neutrophils, activation of PI3K following fMLF has been shown to selectively regulate oxidative burst and actin relocalisation, essential for cell polarisation. The MAP kinase pathway, in particular ERK-1/2, selectively regulates chemokinesis as well as signal transducers and activators of transcription proteins and adaptor proteins (Wenzel-Seifert et al 1998).

Signalling via FPR1 is regulated by two processes, receptor desensitisation and agonist-induced internalisation. Desensitisation is the result of uncoupling of G-protein from the receptor, and can occur either by direct ligation or following activation of similar GPCRs. It has been shown that receptor desensitisation and internalisation is reliant on PKC following both homologous activation of FPR1 or heterologous activation of other GPCRs (Le et al 2001b). After initial activation of FPR1 with fMLF, the receptor rapidly reduces its responses to secondary stimulation with the same agonist; this is termed homologous desensitisation. FPR1 is also susceptible to heterologous desensitisation via ligation of a GPCR receptor, C5aR (complement receptor) or CXCL2 (interleukin-8 receptor), in a concentration-dependent manner (Ali et al 1999). These processes complement the classical idea of concentration-gradient dependent migration by peripheral cells to a site of inflammation.

Apart from the ability of FPR1 to mediate chemotaxis it was also noted that fMLF induced a rapid Ca^{2+} mobilisation (Andersson et al 1986). Although Ca^{2+} has been a robust functional response to fMLF, its biological relevance is still unclear. Ca^{2+} is required for cytoskeleton reorganisation, but phagocytosis and chemotaxis can occur in Ca^{2+} depleted cells (Fu et al 2006).

Functionally, FPR1 is mostly associated with host defence; fMLF has been associated with eliciting shape change, adhesion, phagocytosis, cytokine production, superoxide production and degranulation in phagocytic cells (Selvatici et al 2006).

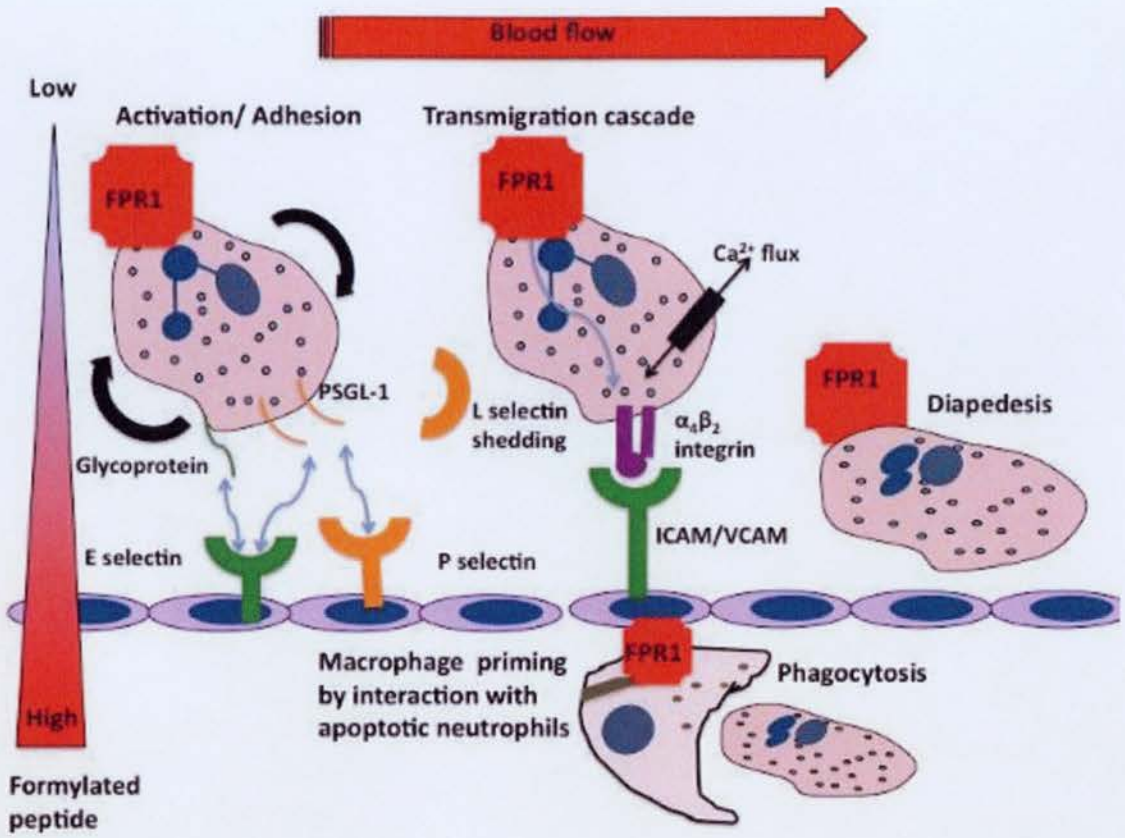


Figure 6. Simplified representation of leukocyte migration cascade via the FPR family (Adapted from Dufton and Peretti 2010). Increasing gradient of formyl peptide leads to a well-characterised sequential response activating circulating and resident leukocytes. FPR1 is activated at low concentration of formyl peptide. FPR activation of leukocytes, results in integrin expression and activation inducing firm adhesion to the endothelial cells. High concentration or repeated stimulation by formyl peptide can lead to receptor desensitisation, which may be a prominent regulatory mechanism. FPRs play a role in macrophage phagocytosis and as yet uncharacterised roles in innate-adaptive cell interactions.

FPR1= Formyl peptide receptor 1; ICAM= intracellular adhesion molecule; PSGL-1= P-selectin glycoprotein 1; VCAM= vascular cellular adhesion molecule.

4.9. FPR1 AND DISEASE

Besides receptor mutations, the degree of FPR1 expression can be modulated in disease. For instance, increased fMLF binding sites are reported on circulating PMN taken from patients suffering from emphysema (Stockley et al 1994), indicative perhaps of an unabated systemic response to bacteria in the lungs that lead to the chronic pathology. Higher binding for the tri-peptide was also shown in patients suffering from Crohn's disease, and this was associated with augmented responses to fMLF (Anton et al 1989). It is plausible that FPR1 expression on the surface of circulating leukocytes could change as result of cell activation, and thus serve as a marker of systemic inflammation.

4.10. CYCLOSPORIN H

Formula: $C_{62}H_{111}N_{11}O_{12}$

Alternate name: 5-(N-Methyl-D-valine)-cyclosporin A

Molecular wt.: 1202.6

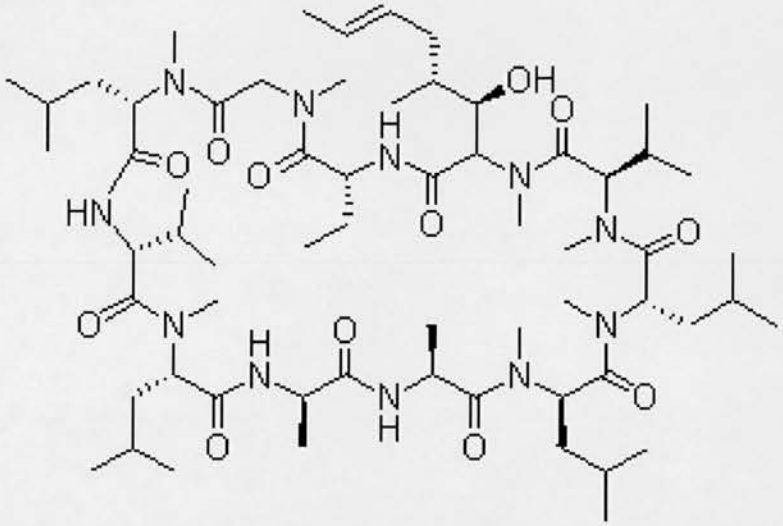


Figure 7. Cyclosporin H

Cyclosporin is an immunosuppressive compound that acts by lowering the activity of T-cells and their immune response. Cyclosporin A (CsA) binds with high affinity to the cytosolic protein cyclophilin of T-cells, a critical step in the molecular mechanism of action of cyclosporins, whereas cyclosporin H, an undecapeptide, (CsH) has extremely low affinity for cyclophilin. CsH differs from CsA by the substitution of the L-methyl valine at position 11 with its D-isomer. CsH is a potent and competitive inhibitor of the formyl peptide receptor. In contrast to CsA, which suppresses early events in the activation of T and B lymphocytes, CsH has no effect (Von Wartburg and Traber 1986, Sigal and Dumont 1992). Studies have shown that CsH is a potent inhibitor of fMLF induced release of O^{2-} from neutrophils (Wenzel-Seifert et al 1991). Interestingly, CsH has no effect on neutrophil chemoattractants C5a, platelet activating factor and LTB_4 (Wenzel-Seifert et al 1991), suggesting that CsH is a selective FPR receptor antagonist. CsH may be a valuable drug to elucidate further the role of endogenous and bacterial formyl peptides in the pathogenesis of inflammatory processes *in vivo* (de Paulis et al 1996). Finally, studies are needed for the development of a new class of anti-inflammatory drugs, i.e., formyl peptide receptor antagonists.

4.11. FURA 2AM

Fura-2-acetoxymethyl ester or Fura-2AM, is a membrane-permeable derivative of the ratiometric calcium indicator Fura-2 used to measure cellular calcium concentrations by fluorescence. When added to cells, Fura-2AM then crosses cell membranes and once inside the cell, the acetoxymethyl groups are removed by cellular esterases. Removal of the acetoxymethyl esters regenerates "Fura-2", the pentacarboxylate calcium indicator (figure 8). Measurement of Ca^{2+}-induced fluorescence at both 340 nm and 380 nm allows for calculation of calcium concentrations based 340/380 ratios. The use of the ratio automatically cancels out certain variables such as local differences in fura-2 concentration or cell thickness that would otherwise lead to artifacts when attempting to image calcium concentrations in cells.

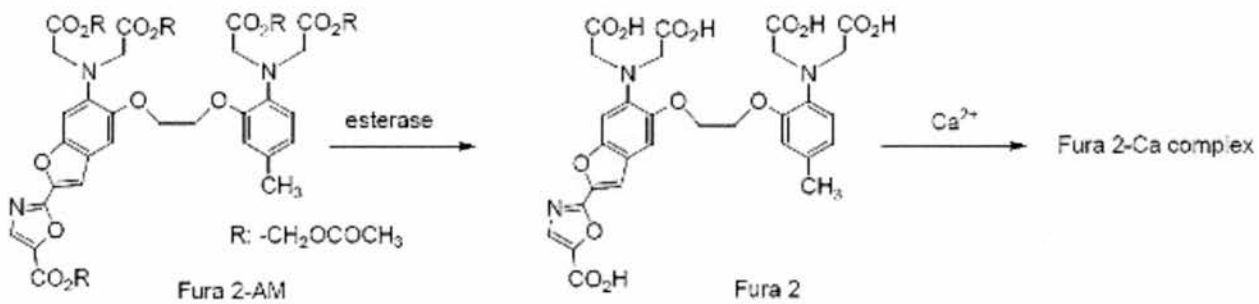


Figure 8. Mechanism by which Fura 2am detects calcium flux.

4.12. RESULTS FROM APOPTOSIS ASSAYS

Dextran sedimentation and discontinuous Percoll gradient, as a means to isolate neutrophils from peripheral venous blood, result in cell purity of ≥95%, with between 1% and 5% eosinophils and 1–2% contaminating mononuclear cells. In individuals with atopic conditions, such as hay fever and asthma, eosinophils can represent >5–10% of the granulocyte population.

Once isolated by Percoll Gradient, peripheral human neutrophils were exposed to the following conditions:

1. Atorvastatin (Sigma Aldrich) at final concentrations of 1nM, 10nM, 100nM, 1μM, 10μM (standard doses that have been used in the literature, Wassmann et al 2002).
2. Atorvastatin 10μM + 100μM Mevalonic acid (standard dose that has been used in the literature, Wassmann et al 2002) (Sigma Aldrich).
3. Mevalonic acid at final concentrations of 10nM, 100nM, 10μM, 100μM.

Flow	Inference
Annexin V negative and PI negative	Live cells
Annexin V positive and PI negative	Apoptotic cells
Annexin V and PI positive	Necrotic cells

Table 1. Interpretation of Annexin V and propidium iodide staining

Apoptosis was assessed by blinded morphological assessment of May–Gründwald–Giemsa stained cytospins, confirmed by quantification of annexin-V–fluorescein isothiocyanate binding and propidium iodide staining.

4.13. CELL COUNTS

At 12 and 20hours, cytocentrifuge preparations from treated and control neutrophil suspensions were fixed and stained. Cells were examined under oil immersion light microscopy and apoptotic cells were defined as cells containing darkly stained pyknotic nuclei (Savill et al 1989). For each time point at least 400 cells were counted from at least 4 different fields.

4.14. STATISTICAL ANALYSIS

Flow cytometry analysis was performed using FlowJo v10.0.4 (Tree Star, Ashland, OR, USA). Results are presented as mean ± standard deviation (SD). Data were analyzed by one-way ANOVA with a Newman-Keuls multiple comparison post hoc test (GraphPad Prism v6; GraphPad Software, La Jolla, CA, USA); significance was accepted with P values: $*P < 0.05$.

4.15. TIME COURSE FOR APOPTOSIS- 12 and 20 hours (N=5)

When cultured under physiological conditions, neutrophils showed a time dependent increase in the number of cells exhibiting the classical morphological features of apoptosis (N% &SD). Apoptosis was confirmed by flow cytometry and by cell counts of cytospins.

When neutrophils were co cultured with atorvastatin, there was a dose and time dependent increase in the number of neutrophils undergoing apoptosis. Atorvastatin failed to induce apoptosis at an earlier time point of 12 hours even at a dose of 10μM. Doses higher than 10μM led to necrosis of cells even at 12 hours, hence maximum dose used was10μM.

At 20 hours, maximum apoptosis was achieved at a dose of 10μM atorvastatin; p=0.0002 (see table 2).

Apoptosis facilitated by atorvastatin at 20 hours, was reversed by co culturing cells with mevalonic acid 100μM.

Treatment	% Viable	% Apoptotic cells	% Necrotic cells
Negative control	67.3(2.1)	29.6 (1.8)	1.2 (0.2)
100nM Atorvastatin	69.6 (0.8)	30.3 (1)	0.6 (0.8)
10μM Atorvastatin	63.8 (9.2)	36.9 (5.1)	0.7 (5.1)
10μM Atorvastatin + 100μM Mevalonic acid	70.0 (1.6)	28.8 (5.6)	1.9 (1.2)
Roscovitine	6.5 (2.7)	83.7 (3.2)	9.9 (0.4)

Table 2. Table showing cumulative flow data of the treatments.

APOPTOSIS FLOW PLOTS AT 20-HOURS

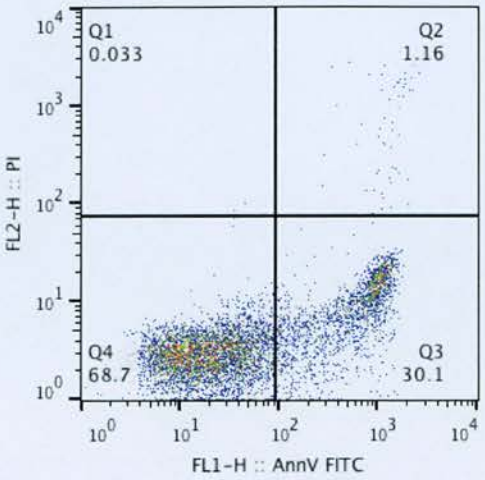


Figure 9a.

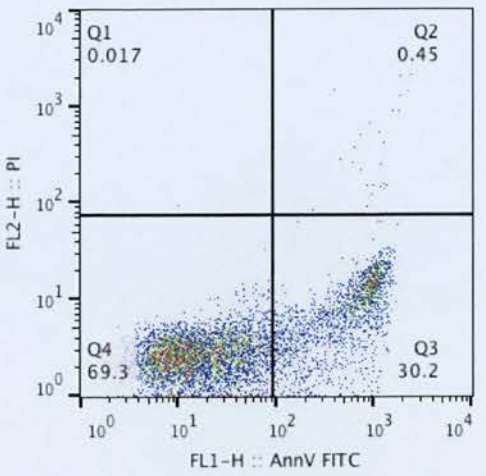


Figure 9b.

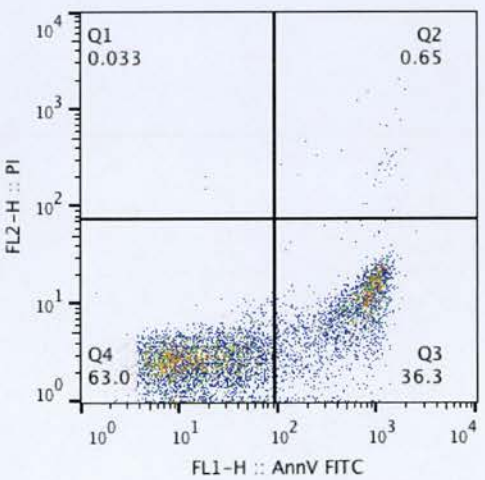


Figure 9c.

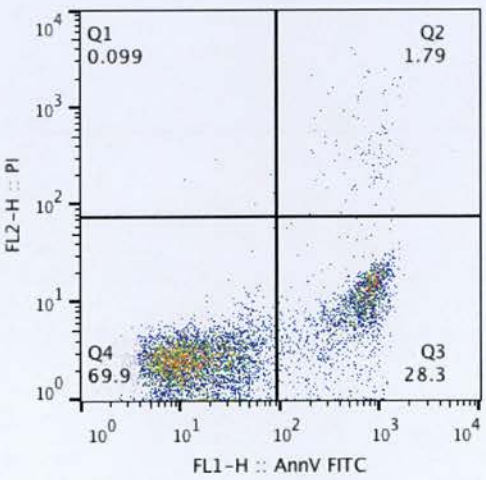


Figure 9d.

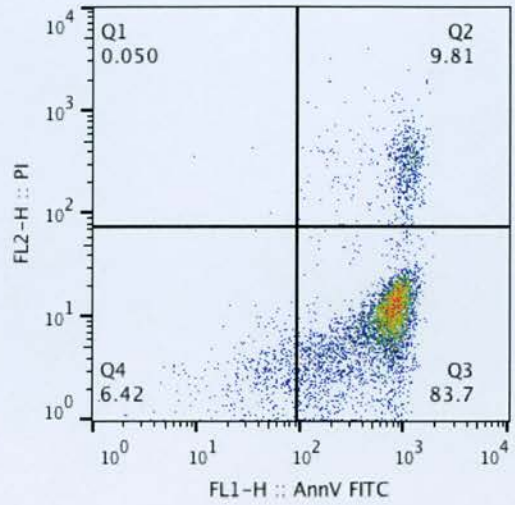


Figure 9e.

Figure 9. Flow plots obtained at 20 hours. 9a= negative control; 9b= 100nM atorvastatin; 9c=10μM atorvastatin; 9d= 10μM atorvastatin+ 100μM mevalonoc acid; 9e= 20μM mevalonic acid

CYTOSPINS OBTAINED AT 20 HOURS

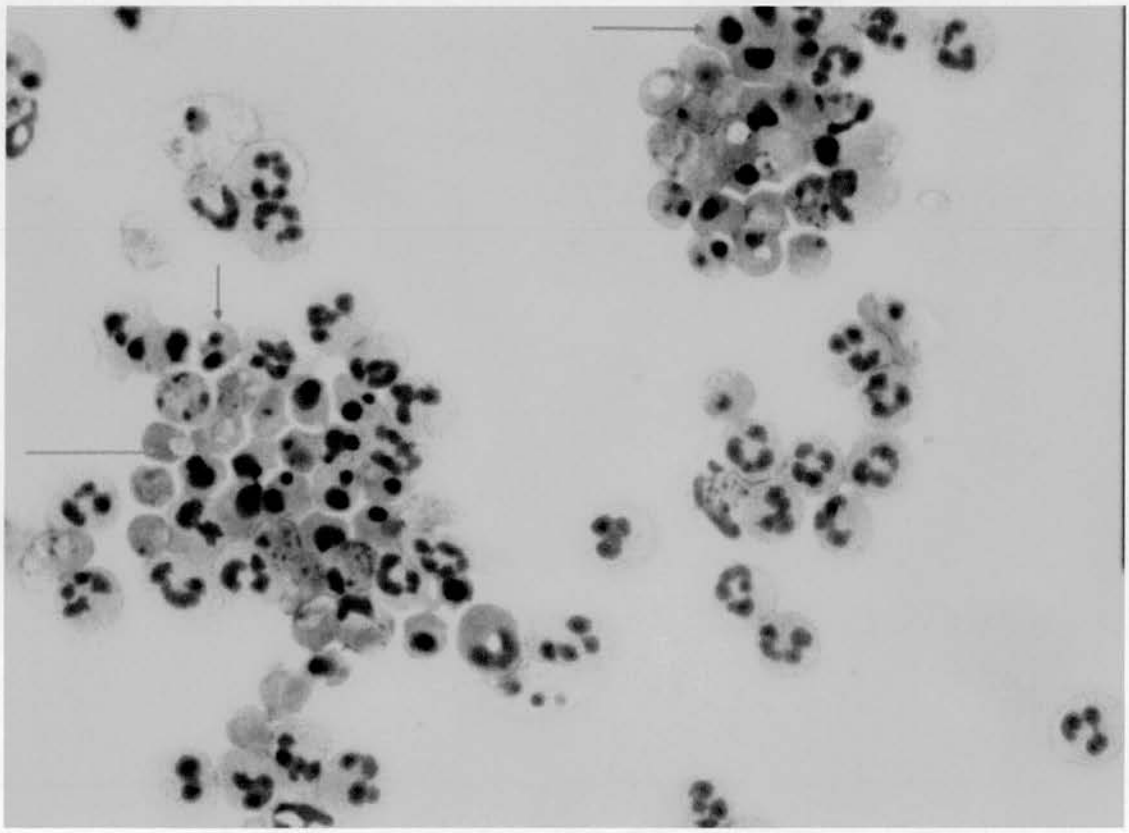


Figure 10. Negative control; red arrows indicate apoptotic neutrophils with pyknotic, darker stained nuclei with intact cell membrane; blue arrows indicate apoptotic cells undergoing secondary necrosis. Note other neutrophils in the field which are viable, have multi lobulated nuclei, intact cell membrane and uniformly stained nuclei.

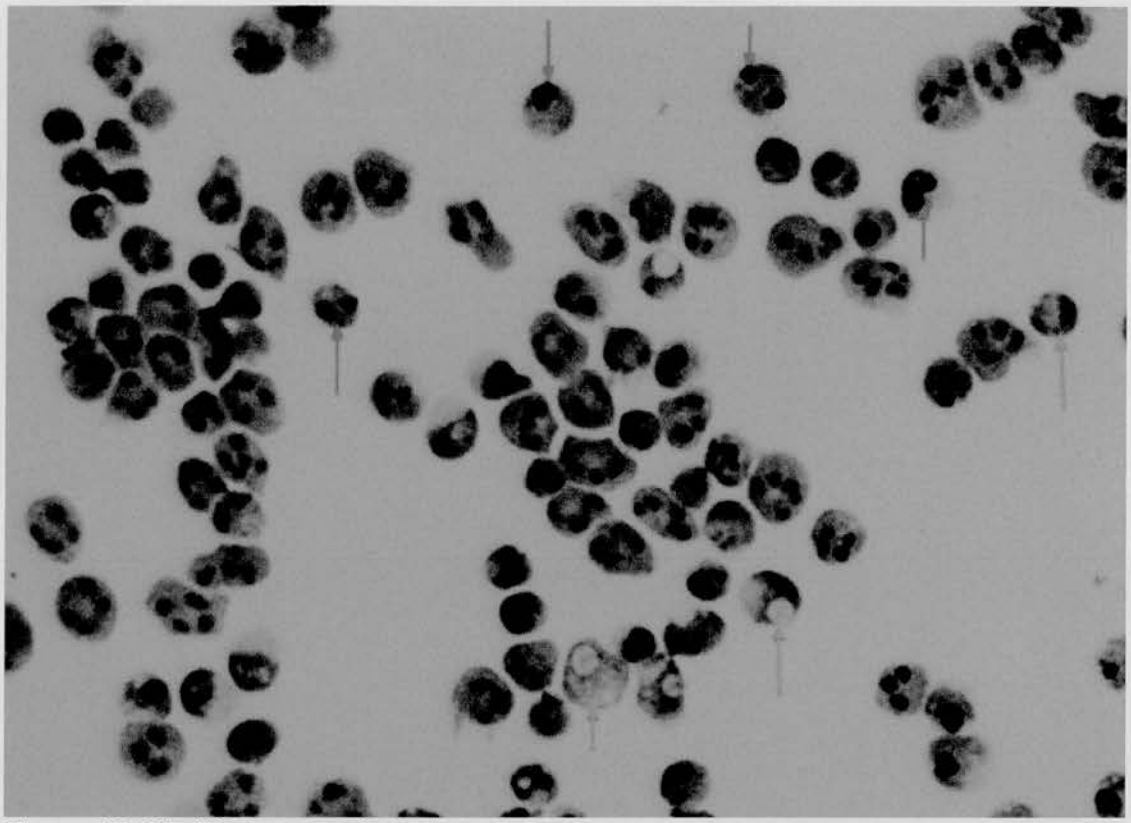


Figure 11. 10μM atorvastatin

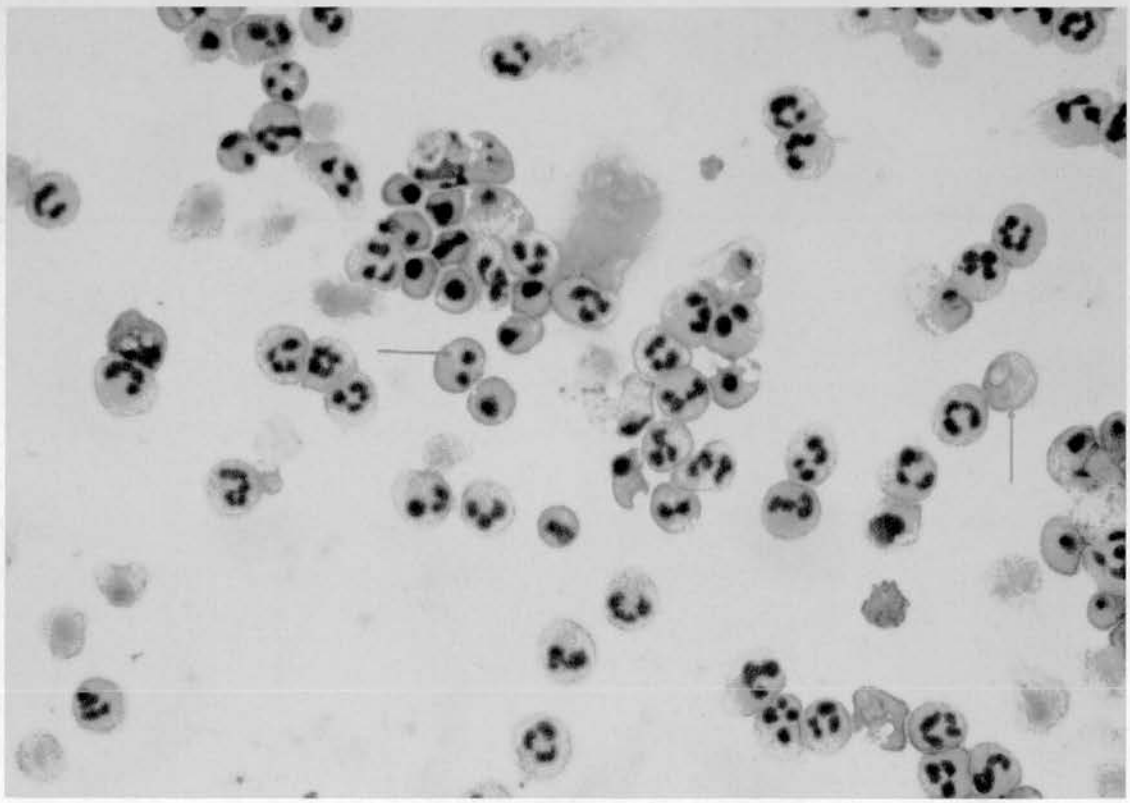


Figure 12. 10μM atorvastatin +| 100μM mevalonic acid

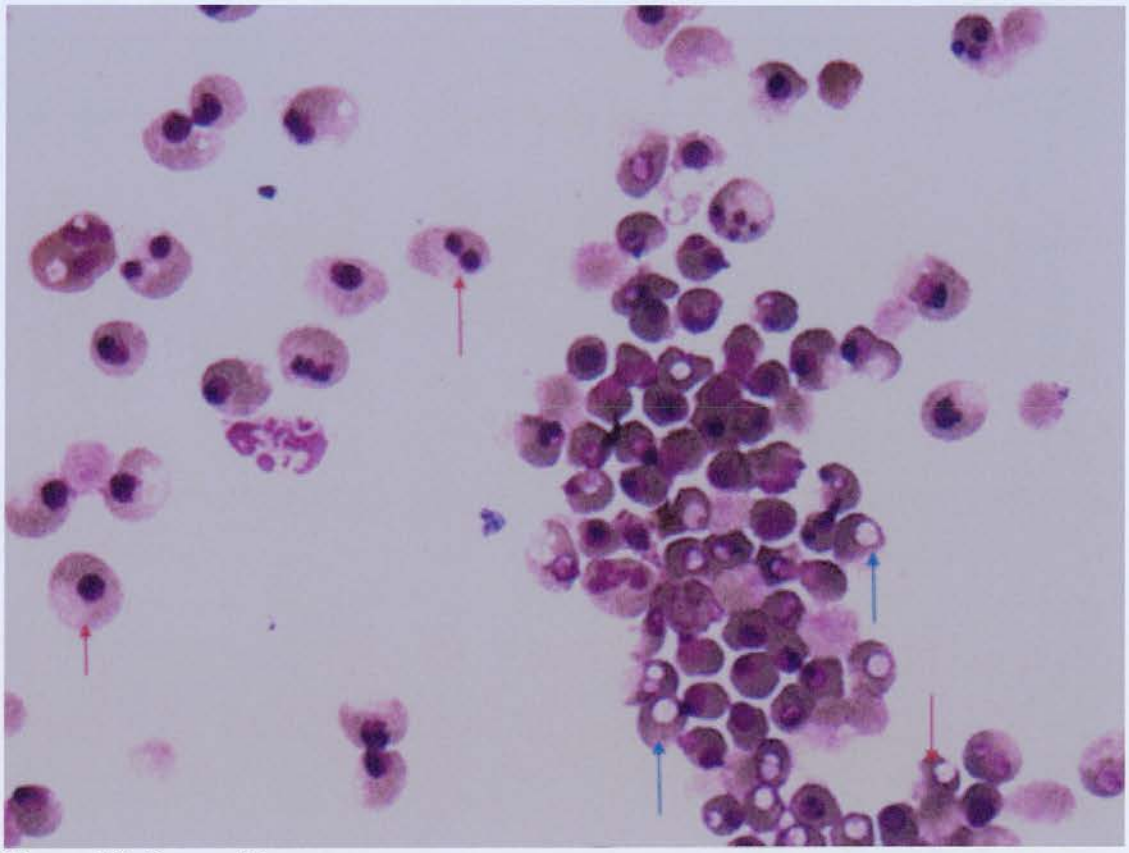


Figure 13. Roscovitine.

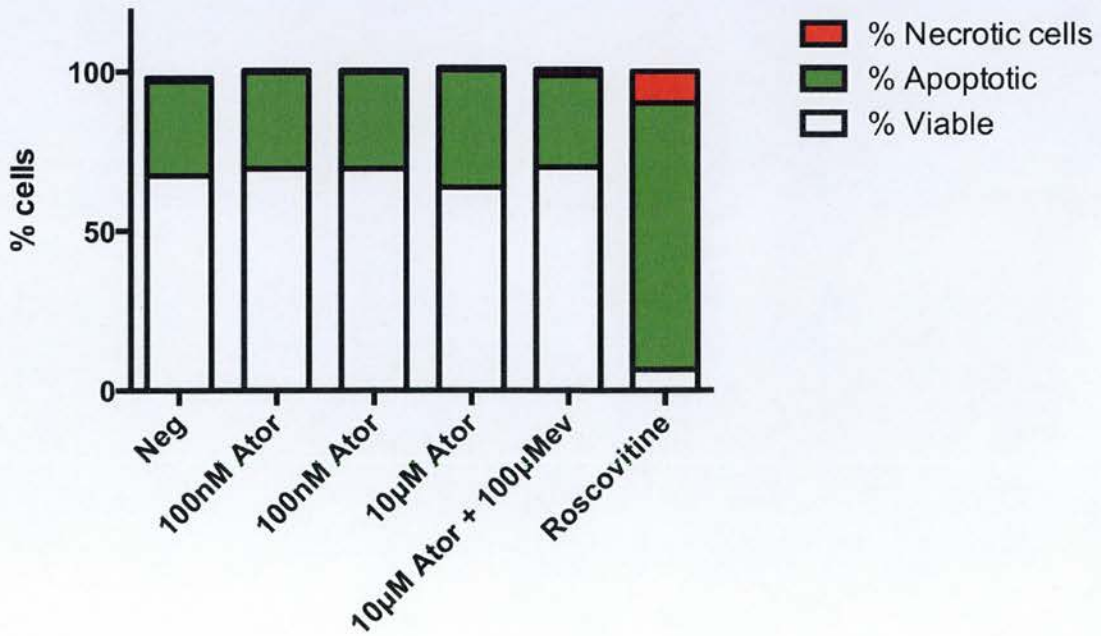


Figure 14. Cumulative data of cytospins at 20 hrs

4.16.CALCIUM FLUX

REAGENTS

- HBSS (Hanks balanced salt solution) without divalent cations-no phenol red
- fura-2/AM
- HBSS without phenol red but with divalent cations.
- 250 mM EGTA ethylene glycol tetraacetic acid (pH 8.0 (diluted in ddH_2O)
- Triton-100 (1%)

After isolation of neutrophils as described above, neutrophils were resuspended (10^7/ml) in HBSS without divalent cations-no phenol red. Cells were then incubated with with fura-2/AM (acetoxymethyl ester) (final concentration 2 μM) for 30 mins at 37 °C (1:1000 dilution). Cells were then washed twice to remove fura-2/AM and leave in HBSS without divalent cations for 10 mins for optimal de-esterification. Neutrophils were then resuspended at 2 x 10^6/ml in HBSS without phenol red but with divalent cations.

The changes in fluorescence were determined upon agonist fMLF stimulation; addition using a fluorimeter, with dual wavelength excitation (340 and 380 nm), emission at 510 nm, fitted with a thermostated cuvette compartment and stirring attachment, to ensure complete mixing of reagents. 2000ml of cells were used in a 3ml cuvette or 500ml cells in 1ml cuvette. Fluorescence was calibrated at start and end of experiment. Maximal fluorescence (R_{max}) and the minimum fluorescence (R_{min}) was obtained. R_{max} is the ratio obtained in the presence of saturating $[Ca^{2+}]i$ [after treatment with 200ml (50ml if small cuvette) Triton-100 (1%). R_{min} is the ratio obtained in the absence of Ca^{2+} (addition of 200ml of 250 mM EGTA (50ml if small cuvette)].

Atorvastatin (at final concentrations of 1nM, 10nM, 100nM, 1μM, 10μM) and 100μM) was added to neutrophils in 1:100 volume.

Cyclosporin H (FPR1 receptor antagonist) was used as a positive control and was added at a final concentration of 1μM.

$[Ca^{2+}]_i$ was calculated from the relationship $[Ca^{2+}]_i = Kd.(R-R_{min})/(R_{max}-R).b$: where $[Ca^{2+}]_i$ is the cytosolic calcium concentration, R is the ratio of fluorescence obtained at 340 and 380 nm in the cuvette before calibration, R_{max} is the fluorescence ratio under saturating $[Ca^{2+}]_i$, R_{min} is the fluorescence ratio in the absence of Ca^{2+}, Kd is the dissociation constant for fura-2/AM, taken as 224 nm at 37°C and b is the fluorescence ratio at 340 nm of cells in the absence and presence of Ca^{2+}.

4.16.1. STATINS AND Ca^{2+} FLUX

(i) fMLF induced Ca^{2+} flux

fMLF (10nM) induced a significant rise in calcium flux and this was inhibited when FPR1 receptor blocker cyclosporin H- 1μM was added simultaneously (positive control). fMLF induced increase in cytoplasmic Ca^{2+} was reduced by addition of atorvastatin (figure 14).

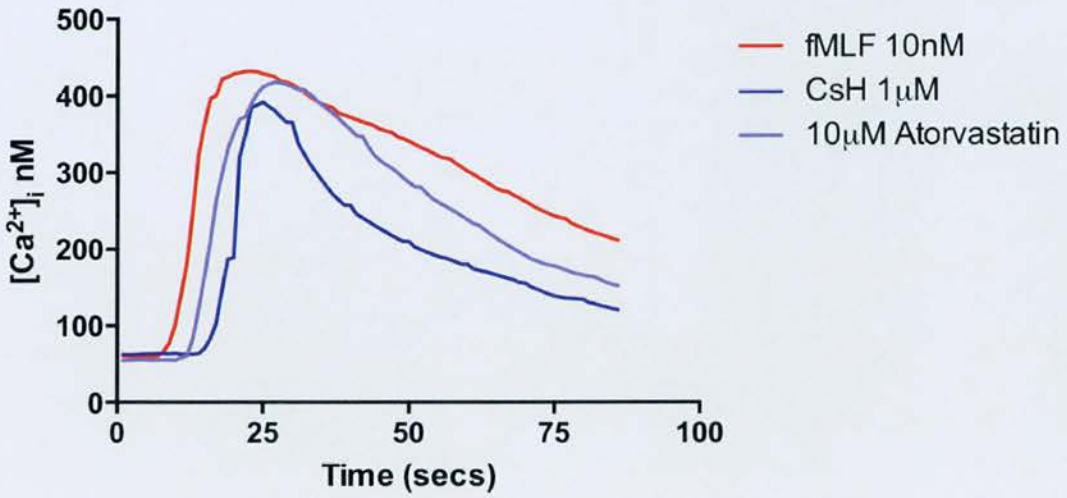


Figure 15. fMLF induced increase in $[Ca^{2+}]_i$ flux; inhibited by Cyclosoprine H and reduced by Atorvastatin.

(ii) Pre incubation with statins

Pre incubation for at least 15 minutes (shown below) was required for statins to reduce fMLF induced increase in $[Ca^{2+}]_i$ flux. There was no effect of statins on $[Ca^{2+}]_i$ flux when added concurrently with fMLF.

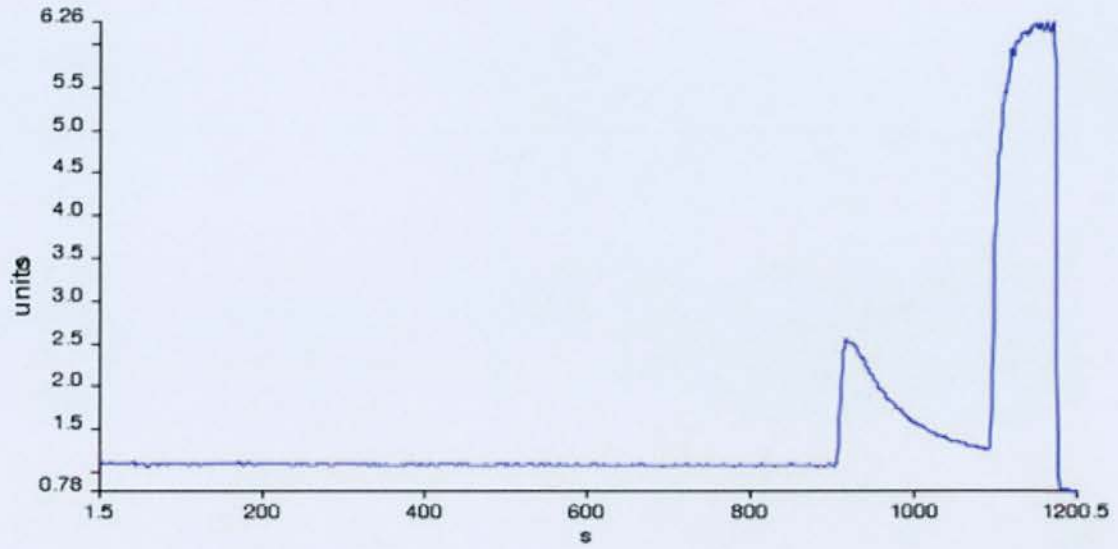
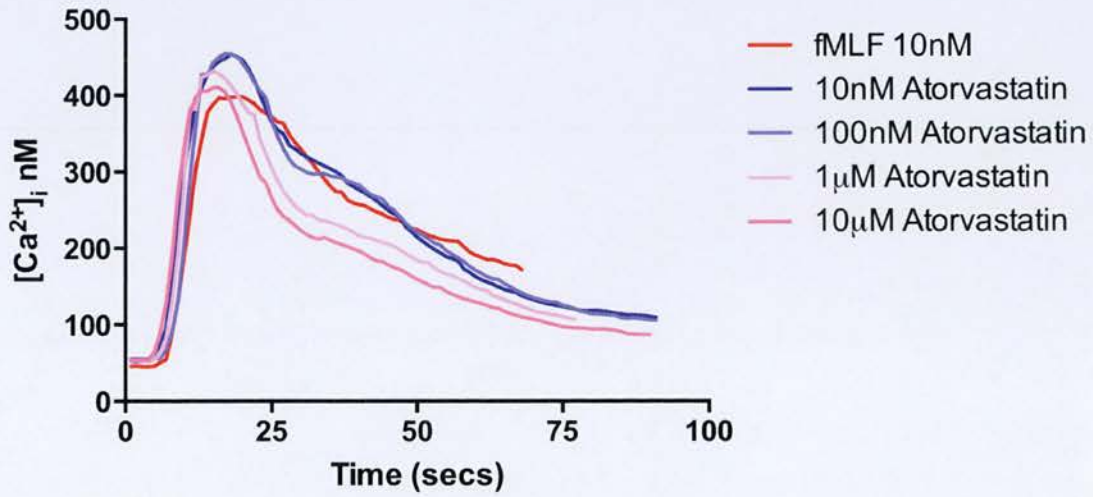


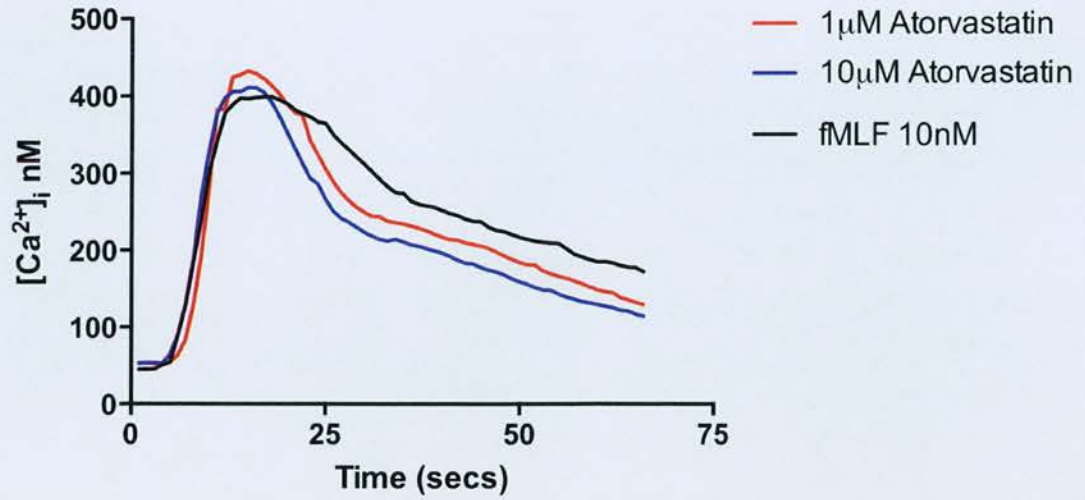
Figure 16. Pre incubation with statins for 900 secs before addition of fLMF.

(iii) Dose response curve with stains

The minimum dose at which statins were able to reduce fMLF induced $[Ca^{2+}]_i$ flux was 10nM. Maximum reduction was achieved at 10μM (see figure 17a&b).



a.



b.

Figure 17 a&b. Atorvastatin dose response curves in fMLP induced $[Ca^{2+}]_i$ flux. Maximum inhibition is obtained at 10μM; AUC= 16888 (fMLF 10nM); 16181 (1μM atorvastatin) and 16134 (10μM atorvastatin); p=0.05 for comparisons.

Hence further studies are needed to establish if statins regulate Ca^{2+} flux by altering the extracellular or intracellular pathways. If statins are regulating extracellular Ca^{2+}mobilization, is this occurring specifically by activation of store operated calcium influx receptors? Alternatively, if statins are mobilizing Ca^{2+} by release from intracellular stores, are statins mediating this response by the G-protein coupled receptors or are the tyrosine kinase receptors? Exploring the role of statins in PAF mediated Ca^{2+} flux, would help assess if statins cause cross receptor desensitization or whether it affects Ca^{2+} flux further downstream.

CHAPTER 5
DISCUSSION

5.1. SUMMARY OF BRONCHIECTASIS

Bronchiectasis is a chronic disabling respiratory condition characterized by chronic cough, chronic sputum production and recurrent chest infections. It is regarded as an orphan disease not because of its rarity but because of the paucity of randomized controlled trials. In a recent PubMed search, only 133 trials have been conducted in bronchiectasis, compared with 3253 for chronic obstructive pulmonary disease, 5405 for lung cancer, and 8748 for asthma (Mandal P and Hill A). The true incidence of bronchiectasis in the modern era of using computed tomography of the chest is not known. We have over 750 patients in Edinburgh, UK monitored in secondary care from a population of about 483,000. Patients frequently utilize primary and secondary care resources through consultations, A&E attendances and inpatient admissions.

The pathogenesis is poorly understood but pulmonary pathology shows excess neutrophilic airways inflammation, but despite this over two thirds of patients are chronically infected with potential pathogenic organisms (Angrill et al 2001). Described by PJ Cole in 1984, there is a vicious cycle of airways inflammation and bacterial infection (Cole 1984). The excessive neutrophilic airways inflammation leads to damage of the bronchial wall and paradoxically promotes more airways inflammation and bacterial infection creating a vicious cycle. Bronchiectasis is a common respiratory condition with a lack of long-term evidence based therapies available. Treatment to date has focused on chest physiotherapy and long-term antibiotics. There are concerns about the use of long term antibiotics in view of antibiotic resistance, side effects and healthcare associated infections. Limitations with the long term use of antibiotics and the exaggerated inflammatory response in the airways, has led to a drive to investigate the efficacy of anti-inflammatory therapies in bronchiectasis.

5.2. CURRENT TREATMENT MODALITIES

To achieve the main treatment goals in bronchiectasis, it is important to break the 'vicious circle' of infection and inflammation. Long term antibiotics and chest physiotherapy is the mainstay of long-term treatment in bronchiectasis. However, there are certain caveats that we have to bear in mind. We are faced the burden of *Clostridium difficile* and Methicillin resistant *Staphylococcus aureus* (MRSA) in addition to antibiotic resistance, with the prolonged use of antibiotics. Consensus on several therapeutic strategies that have been evaluated in cystic fibrosis (CF) and COPD is lacking in bronchiectasis, and a number of controversies need further elucidation. Over the last few years, there has been an international drive to reduce antibiotic usage. There is an urgent need for novel non-antibiotic treatments.

5.3. STATINS IN LUNG INFLAMMATION AND INFECTION MODELS

Cardiologists have used stains for more than two decades for primary prevention of cardiovascular disease. Over the last few years, studies have established that statins have pleiotropic effects, which include modulation of the innate and adaptive immune system and anti-inflammatory properties (Dinarello 2010, Kwak et al 2000). Studies have investigated the role of statins in both animal and human models of sterile and infective inflammation. For example, during sterile inflammation statins attenuate neutrophil recruitment in animal and human experimental systems (Shyamsundar et al 2009, Fessler et al 2005). In an animal model of pulmonary infection with *Staphylococcus aureus*, high dose statin therapy was shown to enhance the formation of extracellular DNA traps by phagocytes within the lung and protect against dissemination of infection (Chow et al 2010, McDowell et al 2011). Boyd *et al*, found that mice treated with prolonged high dose statin, in a model of lung infection with *S. pneumoniae,* had a strong dose dependent effect on protection against *S. pneumoniae* as evidenced by less neutrophil infiltration, maintenance of vascular integrity and less chemokine production (Boyd et al 2012) Additionally, observational studies in community-acquired pneumonia have shown a reduction in 30-day mortality in patients who were on prior statins (Chalmers et al 2008).

We hypothesized that long term statin treatment would improve patients' symptoms through its anti-inflammatory effect consequent on reduced neutrophilic airways inflammation. The aim of our proof of concept study was to establish if atorvastatin could reduce cough, a key feature with clinically significant bronchiectasis.

5.4. STUDY FINDINGS

Six months treatment with high dose atorvastatin led to an improvement in cough from baseline. Cough is one of the cardinal features of bronchiectasis and high dose atorvastatin for 6-months in patients with moderately severe bronchiectasis led to significant reduction of cough, in 40% of patients, the primary end point of this study. In comparison, only 17% had a reduction in cough in the placebo group. There were also significantly reduced IL-8 levels in serum and a significantly increased number of apoptotic airway neutrophils and a trend towards a decreased total number of neutrophils in the sputum in the atorvastatin treated subjects. There was a trend to improvement in exercise tolerance, improvement of 39m more in the statin treated group compared to the group on placebo, but this just failed to reach conventional statistical significance. There was also a trend towards reduction in systemic inflammation (CRP) in the statin treated group, but this did not reach conventional statistical significance. There was no effect on spirometry, airways inflammation, bacterial colonisation or load, quality of life or exacerbations during the study. The study was however not powered for these endpoints. Subanalysis of the data based on compliance to therapy, showed the same results as analysis of the study as a whole. The primary end point was still achieved confirming that although the study was small, the study findings are robust.

The other key study finding was a significant increase in number and proportion of apoptotic neutrophils, decrease in the proportion of viable neutrophils and a trend towards a reduction in the total number of neutrophils, obtained from sputum of patients at the end of 6 months of atorvastatin treatment. We therefore hypothesize that this decrease in the overall number of neutrophils may be related to the altered lifespan of the neutrophils in response to statin treatment.

Surprisingly, there was no reduction in sputum MPO or neutrophil elastase as might be expected. It is expected apoptotic neutrophils would maintain membrane integrity until clearance and have reduced ability to degranulate, generate a respiratory burst, or undergo shape changes in response to external stimuli (Houck 1979). However, release of these granule contents could occur before the induction of apoptosis or

could indicate that there is occurrence of secondary necrosis. This needs to be explored further.

These results are however similar to a study by Llewellyn-Jones *et al*, where indomethacin 75 mg per day was given to 9 patients with clinically stable bronchiectasis (Llewellyn-Jones et al 1995*)*. Pre-treatment with indomethacin, led to a reduction in neutrophil chemotaxis but had no effect on sputum MPO or free elastase activity, suggesting that these measurements in sputum may not accurately reflect airway neutrophil numbers. Further mechanistic studies are needed to assess the immunomodulatory effects of statins on neutrophils.

5.5. ADVERSE EVENTS

There were more dropouts in the statin group compared to the group on placebo (20% compared to 3% in the placebo). The most common cause for dropout was headache and diarrhea (67% of the dropouts), with deranged liver function tests due to statins necessitating withdrawal in one patient. The only dropout from the placebo group was due to personal reasons. However, 80% in the statin group were able to tolerate the high dose statins and complete the full 6-month treatment.

5.6. MECHANISTIC STUDIES

As the key study findings were cough reduction and increase in the number of apoptotic neutrophils and a general trend towards reduction in the number of total and viable neutrophils, our discussion will be focusing mainly on *in vitro* studies done to firstly confirm the RCT findings and then to explore the mechanism by which statins regulate apoptosis.

5.7. APOPTOSIS

Neutrophils are the predominant leucocytes and play a central role in the pathogenesis of bronchiectasis. They amplify the inflammatory process by releasing oxidants and proteases that damage the airways tissues, as well as inflammatory products that leads to recruitment and activation of greater numbers of neutrophils, thereby extending the severity of tissue damage (Kawahito et al 2000).

Neutrophils have the shortest half-life among leukocytes and normally survive for less than a day in the circulation (Liles and Klebanoff 1995) before undergoing morphologic and functional changes characteristic of apoptosis. During culture, 50% to 70% of neutrophils undergo constitutive apoptosis by 20 hours (Ward et al 1999). Apoptosis is a process of regulated cellular death (Akgul 2001) that is mediated by a family of intracellular cysteine proteases or caspases (Hankart 1996) and represents the predominant process responsible for the resolution of the neutrophil-mediated inflammatory response. As neutrophils undergo apoptosis, they lose surface adhesion molecules and the ability to secrete granular contents (Whyte et al 1993) and are subsequently ingested rapidly by macrophages and removed from the area of inflammation with minimal damage to the surrounding tissue. A delayed neutrophil apoptosis could therefore potentially prolong the airway inflammatory response.

5.8. SECONDARY NECROSIS

Morphologically, necrosis is characterized by a disruption of the cellular membrane and a swelling of the cytoplasm and mitochondria, culminating in the complete disintegration of organelles. This is known as primary necrosis (type 1 necrosis). The presence of necrotic cells does not however imply that they did not die by apoptosis. The term secondary necrosis (type 2 necrosis) refers to a process in which late stage apoptotic cells that failed to be engulfed by phagocytes or neighboring cells undergo necrosis. Secondary necrosis is therefore a post-apoptotic event. This process is seen in cultured cells that are undergoing cell death by apoptosis *in vitro*, induced by the absence of survival factor signals or activation of death receptors. These cells, in the absence of phagocytic cells that could engulf them, ultimately cease to be metabolically active, lose membrane integrity, and release their cytoplasmic contents into the culture medium. Secondary necrosis can also occur *in vivo*, in autoimmune disorders associated with impaired clearance of apoptotic cells. In addition, secondary necrosis is seen during massive local apoptosis when macrophages may be unable to cope with the load of apoptotic cells. Therefore, secondary necrosis plays a critical role in inflammation, tissue remodeling, and immune response regulation (Aderem and Underhill 1999).

5.9. Ca^{2+} FLUX

Ca^{2+} ions also serve as important second messengers in signal transduction in many cell types, including neutrophils, leading to the activation of downstream molecules (Lewis 2001). Cytoplasmic levels of Ca^{2+} can increase either from release from internal calcium stores [such as the endoplasmic reticulum (ER) by ionisitol] or by entry from outside the cell via calcium channels, leading to the rapid activation of molecules that promote cell proliferation and cell death (McConkey et al 2001). In neutrophils, stimulation by agonists that bind to the G protein coupled seven transmembrane type receptors such as the fMLF receptor, trigger increases in intracellular Ca^{2+}. Elevation of intracellular free Ca^{2+} levels or mobilization of intracellular Ca^{2+} stores promotes neutrophil longevity (Cousin et al 1997). However, the overall role of calcium in apoptosis is complex and cell specific. Studies have demonstrated that elevation of $[Ca^{2+}]_I$ in neutrophils by calcium ionophores inhibits apoptosis (Whyte et al 1993).

5.10. REGULATION OF THE INFLAMMATORY RESPONSE

5.10.1. Reduction in cough

The primary end point of the study was a reduction in cough and at the end of 6months, 40% of patients in the statin group had a reduction of cough by 1.3 units or more as measured by the Leicester Cough Questionnaire. This compared to a reduction of cough in only 17% of those on placebo for 6 months.

Cough is reported in 90-98% of patients with bronchiectasis (Nicotra et al 1995, King et al 2006). Cough in bronchiectasis is consequent to hypersecretion of mucus and impaired clearance of secretions. The mucus stasis is compounded by the impaired mucociliary clearance, which reflects damaged epithelium. Torrego *et al* demonstrated that bronchiectasis patients have a sensitive cough reflex (to increasing doses of capsaicin). They hypothesized that chronic neutrophilic inflammation and subsequent damage could lead to cough sensitization (Toreggo et al 2006). Studies have shown raised levels of products of neutrophil activation and degranulation such as neutrophil elastase and myeloperoxidase, in bronchoalveolar lavage from bronchiectasis patients (Angrill et al 2001). In addition, high levels of the pro inflammatory cytokines TNF-α, IL-6, IL-8 and LTB_4 have been detected in BAL fluids in bronchiectasis (Wilson 2001, Mikami et al 1998). The presence of bacterial infection is usually associated with more severe inflammation.

As cough is the dominant symptom of bronchiectasis, reduction of cough and measuring its impact on health related quality of life is critical for both disease assessment and targeted management. LCQ is a symptom specific questionnaire and we have validated it for assessing cough severity in bronchiectasis. The mechanism for reduction of cough in our study was not clear, hence we explored this further through *in vitro* studies conducted, based on the other study findings.

5.10.2. Statins and apoptosis

In bronchiectasis, there is prolonged neutrophil persistence that promotes excess airways inflammation (Watt et al 2004). It has been well established that there is a key role for apoptosis, or programmed cell death, in the regulation of inflammation and the host immune response (Rossi et al 2006). *In vivo* models of pneumococcal infection, have demonstrated that inducing apoptosis of neutrophils improved resolution of inflammation and accelerated recovery (Koedel et al 2010).

Although neutrophils appear to be committed to death via apoptosis, it is now clear that the life span and functional activity of mature neutrophils can be extended significantly by pro inflammatory cytokines, including granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interferon gamma, TNF-α and IL-2 (Rossi et al 2006). Increased levels of IL8 and TNF-α have been consistently detected in bronchial secretions in bronchiectasis (Eller et al 1994, Tsang et al 1998). It is therefore likely that high levels of these inflammatory mediators inhibit apoptosis (Dunican et al 2000, Taggart et al 2000), thereby promoting further ongoing inflammation. Hence, it is speculated that in bronchiectasis, the airways tend to enter a positive-feedback loop between prolonged survival and release of pro inflammatory cytokines, leading to further airway inflammation and damage.

Our *in vitro* mechanistic studies showed that statins significantly enhance neutrophil apoptosis at 20 hours and this is dose dependent. We were able to demonstrate this by both flow cytometry and sputum cytospin counts. Rossi *et al* have previously shown that neutrophil apoptosis can be induced by treatment with agents such as CDK inhibitor drugs, with consequent pro-resolution effects (Rossi et al 2006, Leitch et al 2012). Statin induced apoptosis was reversible when neutrophils were co-incubated with mevalonic acid (mevalonic acid is a precursor in the biosynthetic pathway of cholesterol synthesis). However at 12 hours, statins were unable to enhance apoptosis even at a maximum dose of 10μM atorvastatin.

We hypothesize that statin treatment in part regulates neutrophil apoptosis thereby playing an important role in inflammation and resolution.

In a study by Shyamsundar *et al*, they showed that simvastatin has anti inflammatory effects in the pulmonary and systemic compartment in humans exposed to inhaled LPS. They were able to demonstrate that pretreatment with simvastatin for 4days led to an increase in pulmonary neutrophil apoptosis in humans. It is known that during apoptosis, neutrophil secretory processes are shut down and the intact senescent neutrophil is removed by macrophages (Shyamsundar et al 2009). The increase in neutrophil apoptosis was attributed to reduced pulmonary CRP levels. CRP is known to inhibit neutrophil apoptosis (Khreiss et al 2002) and plasma CRP is suggested to have a role in the delayed neutrophil apoptosis seen in cystic fibrosis (McKeon et al 2008). In our study, there was a reduction in CRP although this just failed to reach conventional statistical significance. This needs to be explored further.

Several reports have found that statins reduce the levels of the anti-apoptotic protein Bcl-2 and increased apoptosis and cell death, in human cancer cell lines and murine non-cancer cells (Wood et al 2013). Till date there have been no studies assessing the role of statins on neutrophil apoptosis in a model of chronic airways infection and inflammation. There is also evidence demonstrating that statins enhance efferocytosis *in vitro* and *in vivo*, and that they may play an important therapeutic role in diseases where efferocytosis, a key regulator of inflammation is impaired (Merx et al 2005).

Based on the available data in the literature, we corroborated our study findings and summarized that there was an increase in neutrophil apoptosis, a significant reduction in peripheral IL-8 levels and a reduction in peripheral CRP levels, after 6 months of statin treatment, although this just failed to reach statistical significance. Reduced levels of the pro survival mediator IL-8 in peripheral blood with 6 months statin treatment, could contribute to regulate neutrophil apoptosis, but this needs to be interpreted with caution, as sputum IL8 levels were not reduced. However, we were still unable to explain the mechanism by which statins enhance neutrophil apoptosis in bronchiectasis, but what was clear was that "switching off" of the activated neutrophils and inducing apoptosis has therapeutic potential in bronchiectasis by promoting the resolution of inflammation.

To investigate the mechanism by which statins enhance neutrophil apoptosis, we assessed the role of statins on calcium flux during neutrophil apoptosis. Upon pretreatment with statins for 15 mins, a dose dependent reduction was observed in cytoplasmic calcium levels, measured by spectrofluorimetry of Fura-2-loaded neutrophils, and calcium flux, following stimulation with fMLF.

5.10.3. Apoptosis and Ca^{2+} flux

fMLF increases $[Ca^{2+}]_i$ but the role of fMLF in neutrophil apoptosis is not clear. Colotta *et al* reported that fMLF has no appreciable influence on the *in vitro* life span of neutrophils. Studies have reported fMLF stimulated a considerably greater amount of respiratory burst superoxide release than TNF-α but did not cause significant apoptosis. Lee and colleagues however demonstrated through *in vitro* experiments that the inflammatory mediators fMLF, prolong the functional life span of neutrophils through modulation of apoptosis.

While neutrophils undergo apoptosis constitutively, the rate of apoptosis itself can be modulated. For example, neutrophil apoptosis is delayed by bacterial products and inflammatory mediators, such as fMLF and C5a (Liles and Klebanoff 1995), as well as other receptor-mediated stimuli associated with calcium mobilization and elevation of $[Ca^{2+}]_i$ during neutrophil activation (Ward et al 1999, Whyte et al 1993, Jimenez et al 1997). These data raise an interesting paradox, that apoptosis in the neutrophilis inhibited by stimuli increasing $[Ca^{2+}]_i$ whilst this event has been shown to trigger apoptosis in other cells. McConkey *et al* reported that there was rapid increase in the rate of apoptosis in lymphoid cells with ionophores and the subsequent elevations in $[Ca^{2+}]_i$, were sustained. Neutrophils response to $[Ca^{2+}]_i$ appears to be different. Whyte *et al* demonstrated that increase in $[Ca^{2+}]_i$ in neutrophils is transient but elicits signalling events leading to prolonged inhibition of apoptosis. The role of fMLF and Ca^{2+} in apoptosis is still not clear.

In our studies, we were able to demonstrate that statins reduction in $[Ca^{2+}]_I$ was associated with increase in neutrophil apoptosis *in vitro.* Further studies are needed to explore this pathway further to determine the mechanisms by which statins regulate neutrophil apoptosis.

5.10.4. Statins as an anti inflammatory in infection models

Studies using animal sepsis models have also demonstrated that statins reduce endothelial dysfunction and have anti-thrombotic effects that improve outcome (Tleyjeh et al 2009). In addition, studies using *in vivo* models of pneumococcal infection, have demonstrated that inducing apoptosis of neutrophils improved resolution of inflammation and accelerated recovery (Koedel et al 2010).

In a prospective observational study, Chalmers *et al* showed that in patients admitted to hospital with community acquired pneumonia, prior statin use was associated with significantly lower c rective protein levels on admission and 30-day mortality (Chalmers et al 2008). In a recently published RCT of atorvastatin in critically ill patients admitted to intensive care unit, prior and continued statin use was associated with a an improved survival (Kruger P et al 2013).

We found no adverse effect on viable bacterial load in the sputum of the statin treated patients. There was a trend for fewer patients on statin therapy having three or more exacerbations compared to patients on placebo although this was not statistically significant. Larger multicentre studies are needed to assess exacerbations as the primary endpoint. Our study findings, in addition to the studies listed above, it is perhaps appropriate to conclude that the immunomodulatry effects of statins do not have any detrimental effects on models of acute and chronic lung infection.

The literature supports long-term anti-infective therapies in bronchiectasis and possibly anti-inflammatory therapies using macrolides. Three RCTs using macrolides (2 studies with azithromycin; 1 study with erythromycin) as an anti-inflammatory agent in bronchiectasis have been recently published (Wong et al 2012, Serisier et al 2013, Altenburg et al 2013). All three studies showed that 6-12 month use of

macrolides, either in full dose or lower maintenance dose, led to reduced exacerbation frequencies in bronchiectasis. Here we report the first study exploring the role of statins as a potential anti-inflammatory therapy in bronchiectasis. With 6months statin treatment, there was significant improvement in cough reduction, a decreased serum IL-8 and an increase in airways neutrophil apoptosis. While the mechanism for cough reduction is not entirely clear, we hypothesize that long term statins will enhance apoptosis of sputum neutrophils thereby promoting resolution of inflammation.

5.11. FUTURE PERSPECTIVES

In our 6 months study with atorvastatin in bronchiectasis, we showed that statins reduce cough, enhance neutrophil apoptosis and leads to reduction in systemic inflammation. Further mechanistic studies done following the RCT established that statins enhance neutrophil apoptosis at 20 hours. We were also able to demonstrate that statins reduce fMLF induced increase in $[Ca^{2+}]_i$ and we hypothesize that reduction in calcium flux regulates neutrophil apoptosis, although the mechanism for this needs to be established.

Further mechanistic studies are needed to explore the mechanism by which statins modulate Ca^{2+} flux in neutrophils. Rise in $[Ca^{2+}]_i$ concentrations could be either intracellular or extracellular. As discussed above, rise in $[Ca^{2+}]_i$ comes from release from endoplasmic reticulum by ionisitol triphosphate or from the sarcoplasmic reticulum by cyclic ADP ribose; or influx via plasma membrane Ca^{2+} channels. The most common signaling pathway that increases cytoplasmic Ca^{2+} concentrations is the phospholipase C (PLC) pathway. G protein coupled receptors and the tyrosine kinase receptors activate the PLC pathway by converting membrane phospholipid PIP2 to IP3 and diacylglycerol. IP3 then releases Ca^{2+} from the endoplasmic reticulum. Platelet activating factor (PAF) is a major inflammatory mediator activating neutrophils by increasing $[Ca^{2+}]_i$ concentration. PAF mobilizes cytosolic-free calcium $[Ca^{2+}]_i$ from inositol 1,4,5 triphosphate ($InsP_3$)-sensitive endoplasmic reticulum (ER) calcium stores via a G protein-coupled, phospholipase C-$InsP_3$ pathway. Studies have demonstrated that PAF induced calcium signals are dependent on store operated calcium influx (SOCI) and hence therapeutic interventions blocking SOCI mediated calcium mobilization will modulate inflammatory diseases.

Hence further studies are needed to establish if statins regulate Ca^{2+} flux by altering the extracellular or intracellular pathways. If statins are regulating extracellular Ca^{2+}mobilization, is this occurring specifically by activation of store operated calcium influx receptors? Alternatively, if statins are mobilizing Ca^{2+} by release from intracellular stores, are statins mediating this response by the G-protein coupled receptors or are the tyrosine kinase receptors? Exploring the role of statins in PAF

mediated Ca^{2+} flux , would help assess if statins cause cross receptor desensitization or whether it affects Ca^{2+} flux further downstream.

Neutrophil apoptosis is significantly enhanced following statin treatment (as demonstrated in chapter 4). However, it is not known whether the findings of increasing apoptotic cells in bronchiectatic airways is consequent on uncontrolled apoptosis or failure of clearance of apoptotic neutrophils by macrophages (efferocytosis), thereby also leading to secondary necrosis. Statins have been shown to increase efferocytosis in the murine lung and *ex vivo* in chronic obstructive pulmonary disease alveolar macrophages in an HMG-CoA reductase-dependent manner. However macrophages have not been studied in bronchiectasis and needs to be explored further.

Arguably, the most important enzymes responsible for the cellular events occurring during apoptosis are the caspase (cysteinyl aspartate specific protease) family of enzymes. Of the 14 caspases identified so far, those involved in apoptosis are the initiator caspases (caspase 8,9,10) and effector caspases (caspase 3,6,7) (Murphy et al 2003). The primary function of the effector caspases is the cleavage of key proteins that leads to the characteristic features of apoptotic cells (Nicholson 1999). Do statins regulate apoptosis by modulating the caspase pathway? Further studies assessing the role of statins in caspase inhibition are needed.

5.12. CONCLUSION

Pleoitropic effects of statins have now been well established. In our study, we found that six months of atorvastatin improved cough in clinically significant bronchiectasis patients. There was an increase in the number of airways apoptotic neutrophils suggesting a possible reduction of inflammation and promotion of resolution, thereby impacting on cough.

Multi-centered studies are now needed to assess whether long-term statin therapy can reduce exacerbations. In addition, further studies are needed to assess statin treatment in severe bronchiectasis in patients chronically colonized with *Pseudomonas aeruginosa*.

Mechanistic studies have established that statins enhance neutrophil apoptosis and this is perhaps regulated in part by $[Ca^{2+}]_i$ flux. Further studies are needed to explore this further and establish the mechanism by which statins regulate neutrophil apoptosis.

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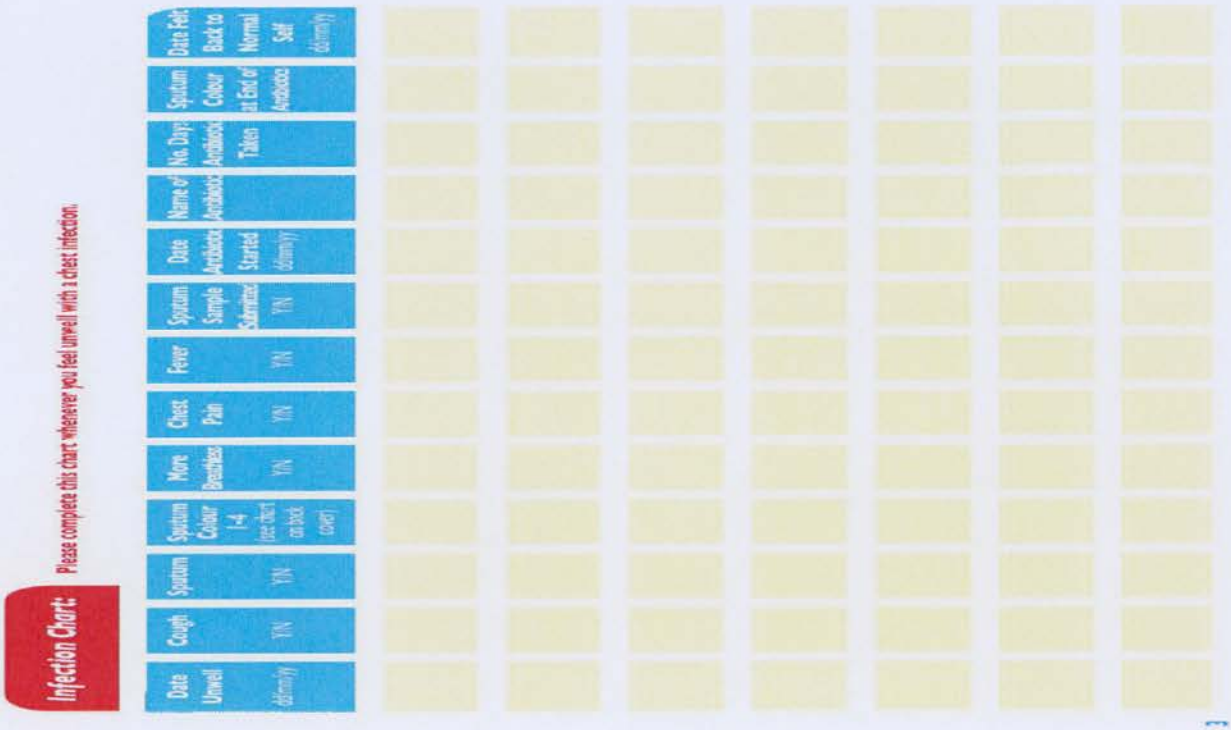
APPENDIX 1

Diary card



APPENDIX 2

Publication from the randomized control trial

Paper currently in press with Lancet Respiratory Medicine

A Proof of Concept Randomized Control Trial of Atorvastatin as a Stable Therapy in Bronchiectasis

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ABSTRACT

Background

Bronchiectasis is characterized by chronic cough, sputum production and recurrent chest infections. The pathogenesis is poorly understood but pulmonary pathology shows excess neutrophilic airways inflammation. Accumulating evidence suggests that statins have pleiotropic effects, including modulation of innate and adaptive immune system and anti-inflammatory effects and therefore is a potential novel anti inflammatory therapy for patients with bronchiectasis.

The aim of our study was to establish if atorvastatin could reduce cough, a key feature in bronchiectasis.

Methods

In this RCT patients aged 18-79 years were recruited. Patients had clinically significant bronchiectasis, which is patients with cough and sputum production when clinically stable, with two or more chest infections in the preceding year and bronchiectasis confirmed on CT scan of the chest.

We excluded: current smokers or ex-smokers of less than 1 year, those with a greater than fifteen pack year history or those with predominant emphysema on CT scan; cystic fibrosis; active allergic bronchopulmonary aspergillosis; active tuberculosis; poorly controlled asthma; pregnancy or breast feeding; known allergy to statins; currently on statins or statin use within 1 year; active malignancy; chronic liver disease; patients on long term oral macrolides; patients chronically colonized with *Pseudomonas aeruginosa*.

Sequence generation was done by block randomization of four, by Tayside pharmaceuticals, NHS Tayside, for 30 patients to receive either atorvastatin 80mg or 30 to receive placebo orally, once daily for 6 months. The placebo (lactose) was not matched to the atorvastatin in appearance. Pharmacy directly dispensed study medications to the patients, hence allocation concealment was maintained at all times to the study investigators. The primary endpoint of this study was a reduction in cough at 6 months compared to baseline as measured by the Leicester Cough Questionnaire (LCQ) score. It is a 19 item self completed quality of life measure of chronic cough which ranges from 3-21, a lower score indicating a more severe cough. The minimum clinically important difference (MCID) is 1.3 Units. The LCQ score is repeatable over 6 months in stable disease. Analysis done was intention to treat. Secondary outcomes included: forced expired volume in one second, forced vital capacity; incremental shuttle walk test; qualitative and quantitative sputum bacteriology; exacerbation frequency; health related quality of life; sputum neutrophil numbers and apoptosis; sputum myeloperoxidase and free elastase activity; sputum-interleukin (IL)-8; systemic inflammation- white cell count, C-reactive protein (CRP) and erythrocyte sedimentation rate, additional systemic inflammatory markers (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and tumor necrosis factor-α) and safety of therapy.

Trial number: NCT01299181; this trial is now completed.

Findings

There was evidence of a difference in baseline to 6-month change in LCQ between the treatment groups, with a significant improvement in the statin treated group, with a mean difference 2.2, 95% CI for difference (0.5, 3.9) p=0.01.

When analyzed as proportion of improvement in LCQ, in the statin treated group 40% patients had a 1.3 Units or more improvement in the LCQ compared with 17% in the placebo group; difference in proportion 23% (95% CI for difference 1%, 45%), p=0.04.

Ten (33%) patients had an adverse event in the statin group compared to three (10%) in the placebo, difference in proportion 23% (95% CI for difference 3%, 43%), p=0.02. There were however no serious adverse events.

Interpretation

In this proof of concept study, six months of atorvastatin improved cough in bronchiectasis. Multi-centered studies are now needed to assess whether long-term statin therapy can reduce exacerbations.

This study was funded by the Chief Scientist Office.

Word Count: 567

INTRODUCTION

Bronchiectasis is a chronic disabling respiratory condition characterized by chronic cough, chronic sputum production and recurrent chest infections. It is regarded as an orphan disease not because of its rarity but because of the paucity of randomized controlled trials- 133 trials in a recent PubMed search, compared with 8,748 for asthma.[1] The true incidence of bronchiectasis in the modern era of using computed tomography of the chest is not known. We have over 750 patients with bronchiectasis in Edinburgh, UK monitored in secondary care from a total Edinburgh population of about 490,000, as per the National Scottish Register 2011. Patients frequently utilize primary and secondary care resources through consultations, A&E attendances and inpatient admissions.

The pathogenesis is poorly understood but pulmonary pathology shows excess neutrophilic airways inflammation, but despite this over two thirds of patients are chronically infected with potential pathogenic organisms.[2] Described by PJ Cole in 1984, there is a vicious circle of airways inflammation and bacterial infection.[3] The excessive neutrophilic airways inflammation leads to damage of the bronchial wall and paradoxically promotes more airways inflammation and bacterial infection creating a vicious cycle. Studies have shown that markers of systemic inflammation including CRP are elevated and correlate directly to disease severity and inversely to lung function and quality of life in stable state bronchiectasis.[4] There is a lack of long-term evidence based therapies available and treatment to date has focused on chest physiotherapy and long-term antibiotics. There are concerns about the use of long term antibiotics in view of antibiotic resistance, side effects and healthcare associated infections. As a result of this and the excess inflammatory response in the airways, there has been a drive to investigate the efficacy of anti-inflammatory therapies.

Statins [3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors] have pleiotropic effects, which include modulation of the innate and adaptive immune system and anti-inflammatory properties.[5,6] For example, during sterile inflammation statins attenuate neutrophil recruitment in animal and human experimental systems.[7,8] In an animal model of pulmonary infection with *Staphylococcus aureus*, high dose statin therapy was shown to enhance the formation of extracellular DNA traps by phagocytes within the lung and protect against dissemination of infection.[9,10] Boyd *et al*, found that mice treated with prolonged high dose statin, in a model of lung infection with *S. pneumoniae,* had a strong dose dependent effect on protection against *S. pneumoniae* as evidenced by less neutrophil infiltration, maintenance of vascular integrity and less chemokine protection.[11] Additionally, observational studies in community-acquired pneumonia have shown a reduction in 30-day mortality in patients who were on prior statins.[12] We hypothesized that long term statin treatment would improve patients' symptoms through its anti-inflammatory effect consequent on reduced neutrophilic airways inflammation. The aim of our proof of concept study was to establish if atorvastatin could reduce cough, a key feature in patients with bronchiectasis.

METHODS

Study Design and Setting

Patients were recruited from South East of Scotland Bronchiectasis Clinic in the Royal Infirmary of Edinburgh, UK from November 2010 to January 2013. We chose atorvastatin as it is a potent statin for reducing cholesterol and we used it at maximum dosage as this is a proof of concept study. Ethical approval was obtained from South East of Scotland Research Ethics Committee.

Randomization

Sequence generation was done by block randomization of four, by Tayside pharmaceuticals, NHS Tayside, for patients to receive either atorvastatin 80mg or placebo orally, once daily for 6 months. The placebo (lactose) was not matched to the atorvastatin in appearance. As pharmacy directly dispensed study medications to the patients, allocation concealment was maintained at all times to the study investigators. The allocation sequence was generated by Tayside pharmaceuticals. The patients were enrolled and assigned to the study by the investigators. The investigators (who also performed the various interventions and assessment) were blinded as the study medications were directly dispensed by pharmacy to the patients. The study statistician analyzed the data. Pfizer, an American based company, manufactures Atorvastatin.

Participants

In this RCT, patients aged 18-79 years were recruited. Patients had clinically significant bronchiectasis, which is patients with cough and sputum production when clinically stable, with two or more chest infections in the preceding year and bronchiectasis confirmed on CT scan of the chest. For bronchiectasis to be diagnosed, the CT scan of the chest was required to show bronchial dilatation, with a bronchus to arterial ratio >1.

We excluded: current smokers or ex-smokers of less than 1 year, those with a greater than fifteen pack year history or those with predominant emphysema on CT scan; cystic fibrosis; active allergic bronchopulmonary aspergillosis; active tuberculosis; poorly controlled asthma; pregnancy or breast feeding; known allergy to statins; currently on statins or statin use within 1 year; active malignancy; chronic liver disease; patients on long term oral macrolides due to the interaction with statin therapy and patients chronically colonized with *Pseudomonas aeruginosa* (defined as two or more isolates of *Pseudomonas aeruginosa* whilst clinically stable in 6 months prior to the study). Patients chronically colonized with *Pseudomonas aeruginosa* were excluded, as they are patients with more severe disease[13,14] and the objective of this study was to investigate atorvastatin in less severe bronchiectasis.

Procedures and Outcomes

The primary outcome of this study is a reduction in cough at 6 months compared to baseline as measured by the Leicester Cough Questionnaire[15] score. We have validated use of this scoring system in bronchiectasis.[16] Cough is a key feature in bronchiectasis and improving this is of major importance to patients with bronchiectasis.

Secondary outcomes included: forced expired volume in one second (FEV_1), forced vital capacity (FVC) and its ratio; incremental shuttle walk test; qualitative and quantitative sputum bacteriology; exacerbation frequency; health related quality of life assessed by the St George's Respiratory Questionnaire; assessment of sputum neutrophil numbers and apoptosis; neutrophil activation in the airway by measuring sputum myeloperoxidase and free elastase activity; neutrophil chemottractant in sputum interleukin (IL)-8; systemic inflammation- measuring white cell count, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), additional systemic inflammatory markers [IL-1β, IL-6, IL-8, IL-10, IL-12p70 and tumor necrosis factor (TNF)-α] -and safety of statin therapy.

Assessments

Assessments below were done at baseline and 6 months. At 3 months, blood tests and compliance was checked (data not presented).

Cough was assessed using the Leicester Cough Questionnaire (LCQ). It is a 19 item self completed quality of life measure of chronic cough which ranges from 3-21, a lower score indicating a more severe cough. The minimum clinically important difference (MCID) is 1.3 Units. The LCQ score is repeatable over 6 months in stable disease (intraclass correlation coefficient of 0.96 (95%CI 0.93-0.97), $p<0.0001$).[16]

An induced sputum using hypertonic (3%) saline for 10 minutes was collected.[17] Each sample was confirmed to be a valid sample suitable for processing if there are >25 polymorphonuclear leukocytes and <10 squamous cells present on Gram stain on low power magnification. 1 ml of the sample was

used for qualitative and quantitative microbiology. Sputum was homogenized and liquefied using an equal volume of dithiothreitol and serially diluted using sterile 0.85% saline to achieve dilutional factors of 10^{-1} to 10^{-4}. *Pseudomonas* isolation agar (Difco), chocolate blood agar containing bacitracin (Oxoid) and horse blood agar (Oxoid) plates were inoculated with 100 µl of dilution. These were incubated at 37°C for 48 hrs. Colonies of the pathogens were then counted to determine the sputum bacterial density, expressed as $\log_{10}$ colony forming units/ml (cfu.ml^{-1}). The rest of the sample was divided equally into portions; the first was treated with 0.1% dithiothreitol to assess total cell numbers and cytocentrifuge samples prepared for total and differential cell counts. Briefly, sputum was washed twice, centrifuged, filtered and cytocentrifuge samples prepared. Cell differential counts determined by counting 400 cells per sample.[18] Apoptosis was confirmed by the colour and shape change of the neutrophil nuclei on the cytospins of sputum samples, as visualized under the microscope. The rest was ultracentrifuged at 30000g for 90 min at 4°C.[19] The sol phase was stored at -70°C until needed for analysing the myeloperoxidase, free neutrophil elastase and IL-8. Myeloperoxidase activity was measured by a chromogenic substrate assay,[20] free elastase activity (NE) was measured spectrophotometrically using the synthetic substrate methoxysuccinyl-ala-ala-pro-valparanitroanilide (MeOSAAPVpNa) (Sigma),[19,21] and IL-8, a key neutrophil chemoattractant in bronchiectasis[22] was assayed using commercially available specific ELISAs (R&D Systems).

Pre bronchodilator FEV_1, FVC and FEV_1/FVC followed by an incremental shuttle walk test were performed (an externally paced, 10 metre incremental field walking test with assessment of dyspnoea before and after using the BORG scale recorded).[23]

Health related quality of life was measured using the St George's Respiratory Questionnaire.[24] It is a 50 item self administered health related quality of life questionnaire with a total score ranges from 0-100; a higher score indicates a poorer health related quality of life. The minimum clinical important difference is 4 units.

Thirty ml of venous blood was collected for full blood count, ESR, CRP, urea, electrolytes, creatinine kinase and liver function tests. 5 ml of blood was centrifuged at 750g for 10 min and the supernatant collected and stored at -70°C, for measuring pro and anti inflammatory cytokines and chemoattractants by cytometric bead array (BD™ Cytometric Bead Array kits).

Side Effects

The presence or absence of side effects was assessed at all study visits. If the alanine aminotransaminase was greater than five times normal or the creatinine kinase greater than three times normal, the study medication was stopped. All side effects were recorded in a patient diary card.

Infective Exacerbations during the Study

Exacerbations were defined as per the BTS guidelines.[25] Exacerbations were treated according to their baseline sputum bacteriology and received 14 days oral antibiotic treatment. Macrolides were not used because of the interaction with statin therapy.

Sample Size

Using a two-sided two-sample test with a 5% level of significance, 90% power, a sample size per group of 27 was needed to detect a change of 1.3 Units in the Leicester Cough Questionnaire. 1.3 Units was chosen as this is the accepted minimum clinically important difference. 30 patients were recruited in each group to allow for dropouts.

Statistical Analysis

All data was analyzed on SAS, Version 9.2. We used an intention to treat analysis for the primary endpoint and a modified intention to treat analysis for the secondary endpoints. For demographic and clinical variables, data are presented as median (interquartile range) for continuous variables and n (%) for categorical variables unless otherwise stated. Baseline to 3 and 6 month change of LCQ was calculated by an unpaired t-test between those receiving atorvastatin versus placebo, as the data was normally distributed. To compare the proportion of patients with clinical improvement as measured by the LCQ or quality of life as measured by the SGRQ a binomial test for the comparison of proportions has been used and differences presented as percentages with accompanying 95% confidence intervals [CI]. Categorical data have been compared between groups using Chi-squared test. A P-value of <0.05 was considered statistically significant for each analysis.

Role of funding source

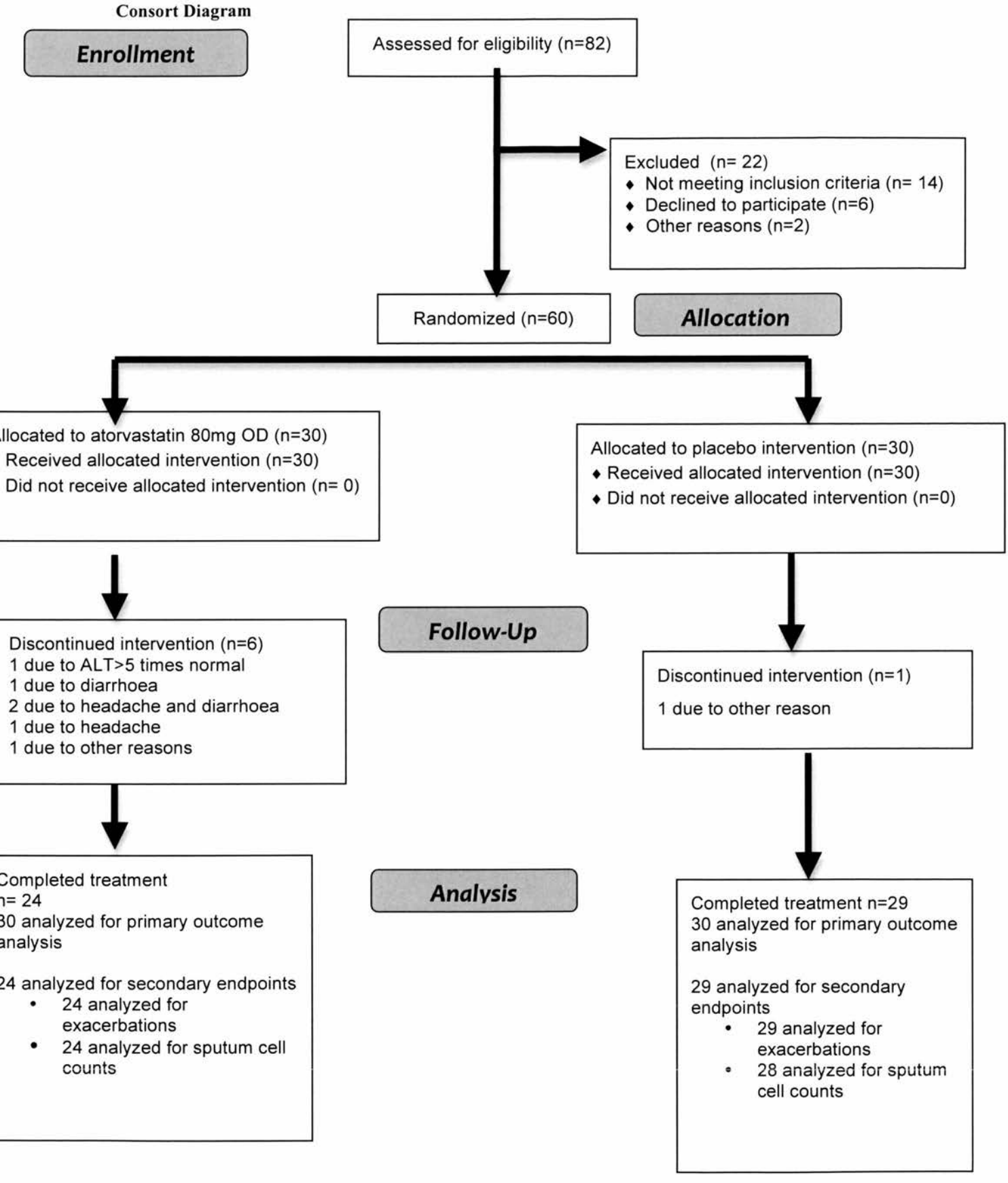
This study is registered with ClinicalTrials.gov, number NCT01299181.

The funding source had no role in the study design, data collection or analysis or in writing the report. The corresponding author had full access to all of the data and the final responsibility to submit for publication.

RESULTS

Baseline Characteristics

Study enrollment started on 26th June 2011. 60 patients were randomized to receive treatment; 30 received active treatment with atorvastatin 80 mg and 30 received placebo. 24 and 29 completed the study in the active and placebo group respectively. 2 patients in the statin group isolated *Pseudomonas aeruginosa* at baseline only and were not chronically infected with *Pseudomonas aeruginosa*.


Consort Diagram
Enrollment
Assessed for eligibility (n=82)
Excluded (n= 22)
♦ Not meeting inclusion criteria (n= 14)
♦ Declined to participate (n=6)
♦ Other reasons (n=2)
Randomized (n=60)
Allocation
llocated to atorvastatin 80mg OD (n=30)
Received allocated intervention (n=30)
Did not receive allocated intervention (n= 0)
Allocated to placebo intervention (n=30)
♦ Received allocated intervention (n=30)
♦ Did not receive allocated intervention (n=0)
Follow-Up
Discontinued intervention (n=6)
1 due to ALT>5 times normal
1 due to diarrhoea
2 due to headache and diarrhoea
1 due to headache
1 due to other reasons
Discontinued intervention (n=1)
1 due to other reason
Analysis
Completed treatment
n= 24
30 analyzed for primary outcome
analysis
24 analyzed for secondary endpoints
• 24 analyzed for exacerbations
• 24 analyzed for sputum cell counts
Completed treatment n=29
30 analyzed for primary outcome analysis
29 analyzed for secondary endpoints
• 29 analyzed for exacerbations
• 28 analyzed for sputum cell counts

Primary Outcome
There was evidence of a difference in baseline to 6-month change in LCQ between the treatment groups, with a significant improvement in the statin treated group, with a mean difference 2.2, 95% CI for difference (0.5, 3.9) p=0.01.
When analyzed as proportion of improvement in LCQ, in the statin treated group 40% (12/30) patients had a 1.3 Units or more improvement in the LCQ compared with 17% (5/30) in the placebo group; difference in proportion 23% (95% CI for difference 1%, 45%), p=0.04 (Figure 1).

At 3 months
There was evidence of a difference in baseline to 3-month change in LCQ between the treatment groups, with a significant improvement in the statin treated group, with a mean difference 3.3, 95% CI for difference (0.9, 5.6) p=0.006.

Sputum Microbiology
Haemophilus influenzae was the most common colonizing organism in both groups at baseline. At the end of treatment, 19/30 (63%) were colonized with microorganisms in the statin group [17/30 (57%) at baseline], compared to 12/30 (40%) in the placebo group [12/30 (40%) at baseline]; for end of treatment proportion. There was no difference in bacterial load when comparing the baseline to 6-month change on the basis of treatment. Mean change in bacterial load was $-2.9x10^7$ ($1.3X10^7$) cfu/ml after 6-months of statin therapy. In the placebo group, mean change in bacterial load at the end of treatment $1.9x10^7$ ($1.2X10^7$) cfu/ml.

Sputum differential count
After 6-months, in the statin treated group, there was an increase in number of apoptotic neutrophils/ 400 cells counted for each sample and a reduction in the total number of sputum neutrophils; table 2 and appendix 1. There were less viable neutrophils and more apoptotic neutrophils. In the placebo group, there was minimal change. There was no evidence of a difference in the number of eosinophils, basophils or monocytes, between active and placebo groups (see table 2).

Sputum inflammatory markers
There is no difference in the baseline to 6month change in IL-8, MPO or free NE between those treated with statin compared to placebo (table 2).

Spirometry
There was no difference in the baseline to 6-month change by treatment for FEV_1, FVC or FEV_1:FVC ratio (table 2).

Exercise Capacity
There was an improvement in the baseline to 6-month change in the statin treated group (table 2).

Systemic Inflammation
There was a reduction in IL-8 in the atorvastatin group (table 2), compared to baseline. However, there was no effect on the IL-1β, IL-6, IL-10, IL12p70 or TNF-α levels (data not shown). In the placebo group, none of the inflammatory markers were significantly reduced.
There was no difference in the baseline to 6-month change in leucocyte count, total neutrophil count or ESR in the treatment group. However, CRP levels decreased in the statin treated group (table 2).

Exacerbation Frequency
8/24 (33%) of patients on statin had 2 or more exacerbations compared to 16/29 (55%) in the placebo group, with a relative risk ratio of 0.6 (95% CI 0.3-1.2).
5/24 (21%) of patients on statin had 3 or more exacerbations compared to 10/29 (34%) in the placebo group, with a relative risk ratio of 0.6 (0.2-1.5).

Quality of life- St. George's Respiratory Questionnaire
There was a mild improvement in the total SGRQ scores in the statin treated group, but not meeting the accepted MCID (a 4 Unit reduction; table 2). There was no difference in the sub scores of the SGRQ (data not shown).

Routine blood tests

There was no difference in the baseline to 6-month change in urea, creatinine, alanine aminotransferase (ALT), or creatinine kinase levels in those treated with statin compared to placebo. However, there was a difference in the baseline to 6-month change in cholesterol in the statin group exhibiting a greater drop, difference in means -1.40, 95% CI (-1.77, -1.02); $p<0.0001$ (table 2).

Comparison of variables where compliant

Using the baseline to 6-month change in cholesterol levels as an indicator of compliance, a stratified analysis was conducted.

In the placebo group, 27/29 did not have a reduction of 1mmol/L point or more in cholesterol and in the atorvastatin group, 15/24 had a reduction of 1mmol/L or more. Within these patients, there is evidence of a difference in baseline to 6-month change in LCQ between the treatment groups, difference 2.2, 95% CI for difference (0.4, 3.9) $p=0.016$.

Comparison of primary outcome where CRP was reduced or remained stable or worsened

Independent of the CRP response, patients on statins had improved LCQ score, see table 2.

Comparison of primary outcome in never smokers

Subanalyses of the data in never smokers in both groups, detected a LCQ improvement in 10 out of 26 patients in the statin group, compared to 2 out of 18 in placebo; 34% difference in proportion (95% CI 13%, 57%); $p=0.04$.

There were 26 never smokers in the statin treated group and 4 ex smokers. All 4 ex smokers dropped out of the study and hence no analysis was done on ex-smokers due to lack of comparative baseline to 6month data.

Adverse Events and Safety

Ten of 30 (33%) patients had an adverse event in the statin group compared to three of 30 (10%) in the placebo, difference in proportion 23% (95% CI for difference 3%, 43%), p=0.02. There were however no serious adverse events.

2/30 (7%) patients in the statin group developed leg pain in the first week of starting the treatment, but this subsided in the second week for both patients. 1/30 (3%) in the statin group and 2/30 (7%) in the placebo group had raised creatinine kinase (CK) while on treatment (<three times upper limit of normal). This was detected at 3 months after starting treatment. These patients had repeat CK levels measured after 1 week and there was either a reduction in the levels or the levels had normalized. No patients had to withdraw from the study due to high CK levels. In the statin group, 1/30 (3%) developed headache, while on treatment and had to withdraw from the study due to it. Diarrhoea was reported in 1/30 (3%) patient within 1 week of starting statin treatment and had to discontinue due to persisting diarrhea. 2/30 (7%) patients developed both diarrhoea and headache whilst on statin treatment and both had to withdraw from the study, due to persisting symptoms. 1/30 (3%) of the patients in both groups developed abdominal discomfort one week after starting treatment, but this improved spontaneously and did not need to stop the trial therapy. At 3 months, 1/30 (3%) patient in the statin group had an ALT of 325 U/L and, as this was >5times normal, the patient was withdrawn from the study. Checking ALT, three days after stopping treatment with statin, showed that it had normalized. 1/30 (3%) patient in the statin group developed haematuria during the first week of starting the treatment. However, he was known to have renal calculi, surgeons reviewed him to confirm this, and he underwent lithotripsy for the renal calculi and continued the study treatment. All patients had normal renal function throughout the study, when measured at baseline and 6-months; table 2.

Adverse events of the study are summarized in table 3.

DISCUSSION

Six months treatment with high dose atorvastatin led to an improvement in cough from baseline. Cough is one of the cardinal features of bronchiectasis and high dose atorvastatin for 6-months in patients with clinically significant bronchiectasis led to significant reduction of cough, in 40% of patients, the primary end point of this study. In comparison, only 17% had a reduction in cough in the placebo group. There were also reduced IL-8 levels in serum and an increased number of apoptotic airway neutrophils and a reduction total number of neutrophils in the sputum in the atorvastatin treated subjects. There was an improvement in exercise tolerance and a reduction in systemic inflammation (CRP) in the statin treated group. There was an association with CRP reduction and LCQ improvement in the statin treated group. In addition, there was an association between statin therapy and exacerbation frequencies with a relative risk reduction in patients with 2 or more exacerbations or 3 or more exacerbations. This exacerbation reduction however did not reach statistical significance but the study was not powered for this end point. There was no effect on spirometry, airways inflammation, bacterial colonisation or load and quality of life during the study. The study was however not powered for these endpoints. Subanalysis of the data based on compliance to therapy, showed the same results as analysis of the study as a whole. The primary end point was still achieved confirming that although the study was small, the study findings are robust.

There was a reduction in systemic IL-8 levels after 6months of atorvastatin. It is known that IL-8 and leukotriene-B_4 (LTB_4) are responsible for the majority to the chemotactic activity of bronchiectatic lung secretions.[25] There was no reduction however in sputum IL-8 levels and hence we are unable to correlate the reduction in systemic IL-8 levels to the other findings in the study.

Immunomodulatory effects of statins have been studied in other chronic lung conditions. Wang *et al* were able to demonstrate that in COPD, prior statin use was associated with reduced exacerbations requiring hospitalization.[26] In addition, long term (>2 years) statin use was associated with a 39% decrease in risk of death in COPD patients.[27] In a large study of 501 lung transplant patients, Li *et al* demonstrated a strong association between postoperative statin administration and improvement of survival, maintenance of graft lung function and slowing of the onset of bronchiolitis obliterans.[28]

In our study, there was an increase in number and proportion of apoptotic neutrophils, decrease in the proportion of viable neutrophils and a reduction in the total number of neutrophils, obtained from sputum of patients at the end of 6months of atorvastatin treatment. We therefore hypothesize that this decrease in the overall number of neutrophils may be related to the altered lifespan of the neutrophils in response to statin treatment. In bronchiectasis, there is prolonged neutrophil persistence that promotes excess airways inflammation.[18] It has been well established that there is a key role for apoptosis, or programmed cell death, in the regulation of inflammation and the host immune response.[29] Although neutrophils appear to be committed to death via apoptosis, it is now clear that the life span and functional activity of mature neutrophils can be extended significantly by proinflammatory cytokines, including granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interferon gamma, TNF-a and IL-2.[29] In contrast, we have shown that neutrophil apoptosis can be induced by treatment with agents such as cyclin-dependent kinase (CDK) inhibitor drugs, with consequent pro-resolution effects.[30,31] In addition, studies using *in vivo* models of pneumococcal infection, have demonstrated that inducing apoptosis of neutrophils improved resolution of inflammation and accelerated recovery.[32] Several reports have found that statins reduce the levels of the anti-apoptotic protein Bcl-2 and increased apoptosis and cell death, in human cancer cell lines and murine non-cancer cells.[33] There is also evidence demonstrating that statins enhance efferocytosis *in vitro* and *in vivo*, and that they may play an important therapeutic role in diseases where efferocytosis, a key regulator of inflammation is impaired.[34]

The role of statins in promoting apoptosis in airway neutrophils or the mechanism by which statins increase apoptotic neutrophils in sputum in bronchiectasis remains to be explored. However, it is clear that "switching off" of the activated neutrophils and inducing apoptosis has therapeutic potential in bronchiectasis by promoting the resolution of inflammation.

Studies using animal sepsis models have also demonstrated that statins reduce endothelial dysfunction and have anti-thrombotic effects that improve outcome.[35] We found no adverse effect on viable bacterial load in the sputum of the statin treated patients. Fewer patients on statin therapy had two or more and three or more exacerbations compared to patients on placebo. This exacerbation reduction

however did not reach statistical significance but the study was not powered for this end point. Larger multicentre studies are needed to assess exacerbations as the primary endpoint.

Surprisingly, there was no reduction in sputum MPO or neutrophil elastase as might be expected. It is expected apoptotic neutrophils would maintain membrane integrity until clearance and have reduced ability to degranulate, generate a respiratory burst, or undergo shape changes in response to external stimuli.[36] However, release of these granule contents could occur before the induction of apoptosis. These results are similar to a study by Llewellyn-Jones *et al*, where indomethacin 75 mg per day was given to 9 patients with clinically stable bronchiectasis.[37] Pre-treatment with indomethacin, led to a reduction in neutrophil chemotaxis but had no effect on sputum MPO or free elastase activity, suggesting that these measurements in sputum may not accurately reflect airway neutrophil numbers. Further mechanistic studies will be needed to assess the immunomodulatory effects of statins on neutrophils.

The literature supports long-term anti-infective therapies in bronchiectasis and possibly anti-inflammatory therapies using macrolides. Four RCTs using macrolides (3 studies with azithromycin; 1 study with erythromycin) as an anti-inflammatory agent in bronchiectasis have been recently published.[38-41] All studies showed that 6-24 month use of macrolides, either in full dose or lower maintenance dose, led to reduced exacerbation frequencies in bronchiectasis. To the authors' best knowledge, this is the first study exploring the role of statins as a potential anti-inflammatory therapy in bronchiectasis. With 6months statin treatment, there was significant improvement in cough reduction, a decreased serum IL-8 and an increase in airways neutrophil apoptosis. While the mechanism for cough reduction is not entirely clear, we hypothesize that long term statins will enhance apoptosis of sputum neutrophils thereby promoting resolution of inflammation.

The major risk factor of long term statin use is myositis and deranged liver function tests.[42] In our study we used the maximum dosage of statin and hence more side effects were anticipated. 5 (17%) did not tolerate statins due to headache, diarrhoea and deranged liver function tests.
There were more dropouts in the statin group compared to the group on placebo (6 (20%) compared to 1 (3%) in the placebo), the most common cause for dropout was headache and diarrhoea. There were no dropouts due to myositis but deranged liver function tests necessitated withdrawal of one patient. The only dropout from the placebo group was due to personal reasons.
24 (80%) in the statin group were able to tolerate the high dose statins and complete the full 6-month treatment. Baseline liver functions need to be recorded when starting patients on statins. Liver function tests need to be checked at 3months and at 1year after commencing treatment or earlier if there is any indication to do so. Although there were no cases of withdrawal due to myositis in the study, patients should be encouraged report symptoms of myositis necessitating monitoring of creatinine kinase once reported.[42]

Limitations
This is a small proof of concept study that was not powered for any of the secondary end points. There was not an exact matching of the active treatment and placebo. The researchers were all blinded to treatment and the study pharmacist dispensed the study medications. We have no reason to believe that the patients were not blinded. Although there was fixed block randomization of four that was used, the investigators were not aware of the treatment, as the allocation was done by an external source. Some patients had a smoking history but we excluded patients with greater than 15 pack year smoking history and evidence of significant emphysema on CT of the chest, making significant COPD unlikely.

Conclusions
Six months of atorvastatin improved cough in bronchiectasis patients. There was an increase in the number of airways apoptotic neutrophils suggesting a possible reduction of inflammation and promotion of resolution, thereby impacting on cough.
Multi-centered studies are now needed to assess whether long-term statin therapy can reduce exacerbations. In addition, further studies are needed to assess statin treatment in severe bronchiectasis in patients chronically colonized with *Pseudomonas aeruginosa*.

Research in context panel

Systematic Review

Bronchiectasis is a chronic disabling respiratory condition characterized by chronic cough, chronic sputum production and recurrent chest infections. There is evidence from *in vitro* studies to suggest that statins have immunomodulatory properties. In particular statins have been shown to reduce neutrophil activation in healthy volunteers challenged to sterile inflammation. International cohort studies in COPD, pneumonia and lung transplantation have demonstrated that prior statin therapy was associated with improved outcome. Bronchiectasis is a neutrophilic inflammatory disease where the driver for persistent inflammation remains unknown but infection is hypothesized to play a major role. There has been an international interest in anti inflammatory therapy in bronchiectasis. Available evidence suggests that statins may have a role in modulating the inflammatory response in bronchiectasis. A systematic review was carried out on any paper before November 2010 on Pubmed, Medline and Embase using the terms 'bronchiectasis,' 'COPD', 'emphysema', 'pneumonia' and 'atorvastatin' or 'statin' and 'randomized control trial' before conducting the study. Only articles in English were taken. There were no positive results. To the authors best knowledge, no previous studies assessing the role of statins in bronchiectasis have been carried out.

Interpretation

Six months of atorvastatin improved cough, a major symptom in patients with bronchiectasis. A potential mechanism found was that there was an increase in the number of airways apoptotic neutrophils, suggesting a possible reduction of inflammation and promotion of resolution, thereby impacting on cough. Although not statistically significant, there was also a relative risk reduction for 2 or more exacerbations and 3 or more exacerbations. This proof of concept study however was not powered to address exacerbations and multi-centered studies are now needed to address whether long-term statin therapy can reduce exacerbations.

Conflict of interest

The authors declared no conflicts of interest.

Author contributions

PM conducted the study, analyzed data and wrote the manuscript.
JDC involved in study design and manuscript preparation.
CG involved in study design, data analysis and manuscript preparation.
CH involved in data analysis and manuscript preparation.
MKS, CD and JWG involved in microbiology analysis and manuscript preparation. DJD and AGR involved in supervising experiments, data interpretation and manuscript preparation.
TS involved in study design and manuscript preparation.
ATH designed the study, PI for the study and manuscript preparation.

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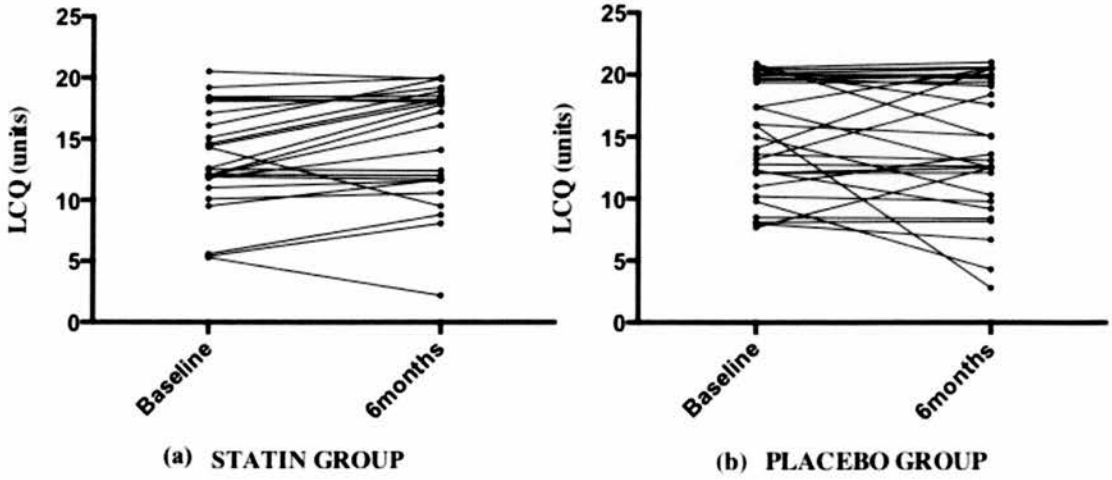
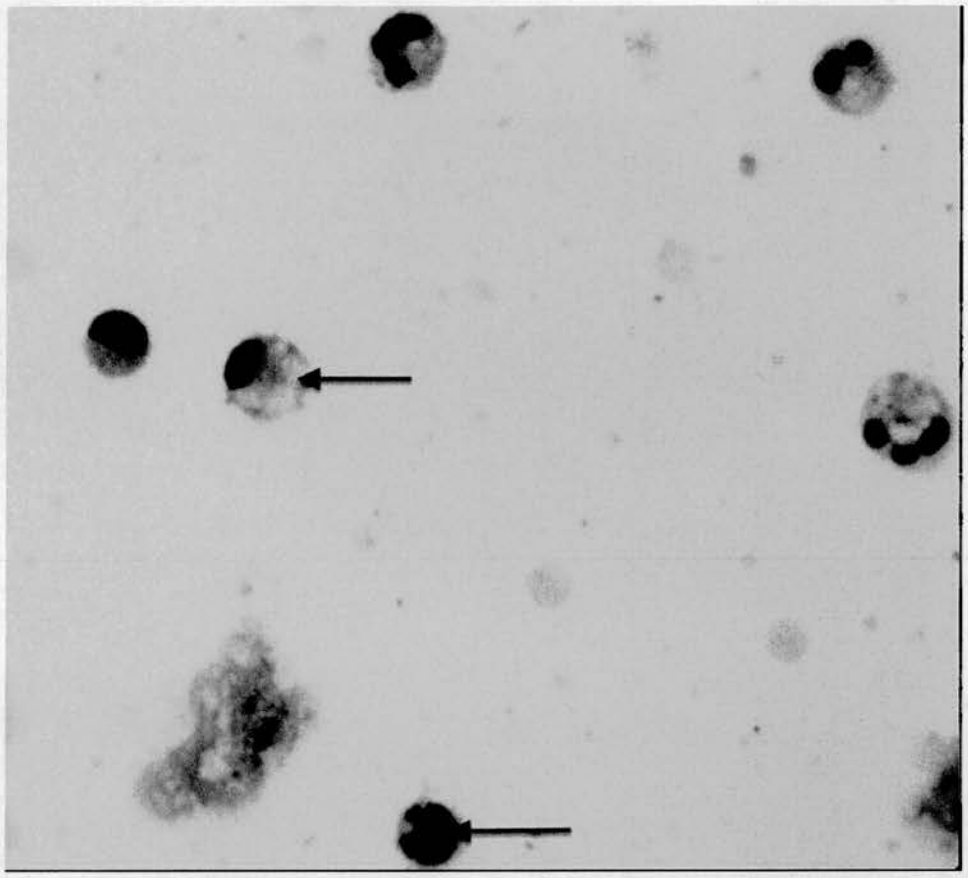


Figure 1a&b: Reduction of cough in the statin group, as measured by LCQ; *p=0.01 for comparison of reduction in cough between both groups, at the end of 6months.



Appendix 1. Increased levels of sputum neutrophil apoptosis following atorvastatin. Figure shows neutrophils (X1000) from sputum (per 400 cells counted) in a patient after 6-months statins. Apoptotic neutrophils indicated by arrows.

	Group receiving atorvastatin 80mg Mean (SD) N=30	Group receiving placebo Mean (SD) N=30	Missing data
Age (years)	60.2 (10.7)	59.1 (11.4)	-
Gender (% female)	17 (57%)	14 (47%)	-
Smoking status **Never** **Ex**	 26 (87%) 4 (13%)	 18 (60%) 12 (40%)	-
BMI (Kg/m^2)	28.8 (8)	28.1 (6.3)	-
Aetiology			-
Idiopathic	21 (70%)	21 (70%)	
Post infectious	4 (13%)	4 (13%)	
Auto immune disease	4 (13%)	4 (13%)	
Inflammatory bowel disease	1 (3%)	0	
IgG_2 deficiency	0	1 (3%)	
High BMI >30Kg/m^2	11 (37%)	10 (33%)	
IHD	2 (7%)	1 (3%)	
Asthma	19 (63%)	17 (57%)	
Previous malignancy	1 (3%)	1 (3%)	
Diabetes mellitus	1 (3%)	1 (3%)	
Respiratory physiology			-
Pre therapy FEV_1 (L)	2.1 (0.8)	2.2 (0.9)	
FEV_1 (% predicted)	78.3% (23.8%)	73.9% (24.5%)	
Pre therapy FVC (L)	3 (1.1)	3.2 (1.1)	
FVC (%predicted)	94.8% (4.2%)	86.4% (25.6%)	
FEV_1 /FVC	69% (0.13%)	69.6% (0.12%)	
Markers of systemic inflammation			3
White cell count ($X10^9$/L)	7.2 (2.2)	6.7 (1.9)	
Neutrophils ($X10^9$/L)	4.3 (1.9)	3.9 (1.1)	
ESR (mm/hr)	15.1 (11.7)	14.8 (10.7)	
CRP (mg/L)	9.9 (15.5)	6.4 (7.7)	
Cholesterol levels			-
Cholesterol (mmol/L)	5.1 (1.1)	5 (0.9)	
Sputum microbiology#			1
Potentially pathogenic microorganisms	17 (57%)	12 (40%)	

Mixed normal flora	13 (43%)	17 (57%)	
No sputum produced	-	1 (3%)	
Other pretreatment medications			-
Inhaled corticosteroids	22 (73%)	18 (60%)	
Oral steroids	0%	2 (7%)	
Long term antibiotic for chest (penicillin)	1 (3%)	1 (3%)	
% DM requiring insulin	0%	0%	
Leicester Cough Questionnaire			-
Units	13.1 (4.0)	15.1 (4.4)	

Table 1. Baseline characteristics of study population. BMI = body mass index; CRP= C-reactive protein; FEV_1= Forced expiratory volume in 1 second; FVC= Forced vital capacity; IgG_2= Immunoglobulin G_2; IHD=ischemic heart disease.

Breakdown of microorganisms isolated at baseline (*Statin group, **Placebo group)

Haemophilus influenzae *8(27%); **6(20%); *Streptococcus pneumoniae* *4(13%); **1(3%); *Staphylococcus aureus* *3(10%); **2(7%); Other enteric gram negative organisms *3(10%); **2(7%); *Pseudomonas aeruginosa* *2(6%); *Moraxella catarrhalis* **1(3%). Some patients isolated more than 1 organism.

	Median (interquartile range) change [6-month to baseline]		Missing data
Outcome	**Atorvastatin N=24**	**Placebo N=29**	
***Sputum differential count* (per 400 cells counted)**			1
Apoptotic neutrophil	5.5 (0.0, 15.0)	0.5 (0.0, 3.0)	
Apoptotic neutrophil (% difference of apoptotic neutrophils)	15.14% (3%)	-1.4% (0.8%)	
Neutrophil	-32.5 (-88.5, 1.5)	-0.5 (-25, 5)	
Neutrophil (% difference of viable neutrophils)	-19.5% (5%)	1.4% (0.6%)	
Basophil	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	
Eosinophil	0.0 (0.0, 0.0)	0.0 (-0.5, 1.0)	
Monocytes	-1 (-2.5, 1)	0.0 (0.0, 2)	
Sputum inflammatory markers			1
Interleukin-8 (pg/ml)	254 (-968, 1075)	260 (-312, 752)	
Myeloperoxidase (pg/ml)	0 (-8.5, 32.5)	0 (-21, 56)	
Neutrophil elastase (pg/ml)	0 (-638, 194)	0 (-1034, 360)	
Spirometry			-
FEV_1 (L)	-0.01 (-0.13, 0.13)	0.06 (-0.06, 0.17)	
FVC (L)	-0.08 (-0.3, 0.13)	-0.07 (-0.34, 0.2)	
FEV_1: FVC	0.01 (-0.02, 0.07)	0 (-0.04, 0.07)	
Exercise capacity			-
Distance walked (m)	35 (-10, 95)	0 (-20,40)	
Exacerbation N (%)			-

0	6 (25%)	9 (31%)	
1	10 (42%)	4 (14%)	
2	3 (13%)	6 (21%)	
≥3	5 (21%)	10 (34%)	
Systemic inflammation			3
White blood cells ($X10^9$/L)	-0.4 (-0.7, 0.5)	-0.1 (-0.8, 0.9)	
Neutrophils ($X10^9$/L)	-0.0 (-0.4, 0.4)	-0.1 (-0.5, 0.9)	
Lymphocytes ($X10^9$/L)	-0.1 (-0.3, 0.1)	-0.0 (-0.2, 0.1)	
Monocytes ($X10^9$/L)	-0.0 (-0.1, 0.1)	-0.1 (-0.1, 0.0)	
Eosinophils ($X10^9$/L)	-0.1 (-0.1, 0.0)	-0.0 (-0.1, 0.1)	
CRP (mg/L)	-1.0 (-6.0, 0.0)	0 (-3.0, 1.0)	
ESR (mm/hr)	-1.0 (-8.0, 1.0)	1.0 (-4.0, 6.0)	
IL-8 (pg/ml)	-1.9 (-0.4, 18.8)	0.3 (-4.2, 7)	
Routine blood tests			3
ALT (U/L)	2.0 (-5.5, 5.5)	-1.0 (-5.0, 4.0)	
CK (U/L)	-5.5 (-49.0, 21.5)	-20.0 (-61, -3.0)	
Cholesterol (mmol/L)	-1.3 (-2.1, -0.6)	0 (-0.2, 0.4)	
Urea (mmol/L)	0.3 (-0.9, 1.1)	0.2 (-0.8, 0.9)	
Creatinine (µmol/L)	-1.0 (-4.0, 3.0)	-1.0 (-4.0, 7.0)	
SGRQ			-
Units	-1.3 (-4.2, 0)	0 (-1.6, 1.1)	
LCQ score (Units) analyzed by CRP response			3

CRP reduction ≥1mg/L	2.5 (0.1, 3.8)	0.1 (-0.2, 0.4)	
No or CRP ≤ 1mg/L	0.55 (-0.2, 2.2)	-1.3 (-4.9, 0)	

Table 2. Summary statistics of secondary end points and adverse effects. ALT= alanine aminotransferase; CK= creatinine kinase; CRP= C-reactive protein; ESR= erythrocyte sedimentation rate; FEV_1= Forced expiratory volume in 1 second; FVC= Forced vital capacity; SGRQ= St. George's Respiratory Questionnaire.

Adverse events	**Statin group N=30**	**Placebo group N=30**
Leg pain	2 (7%)	0
Raised CK	1 (3%)	2 (7%)
Headache	3 (10%)	0
Diarrhea	3 (10%)	0
Abdominal discomfort	1 (3%)	1 (3%)
Deranged LFTs	1 (3%)	0
Haematutria	1(3%)	0

Table 3. Adverse events during the study.